AD_____

Award Number DAMD17-96-1-6026

TITLE: Role of Stroma-Derived Extracellular Matrix in Regulation of Growth and Hormonal Responsiveness of Normal and Cancerous Human Breast Epithelium

PRINCIPAL INVESTIGATOR: Terry L. Woodward

CONTRACTING ORGANIZATION: Michigan State University
East Lansing, Michigan 48824-1101

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Sep 97 - 31 Aug 98)

4. TITLE AND SUBTITLE
Role of Stroma-Derived Extracellular Matrix in Regulation of Growth and Hormonal Responsiveness of Normal and Cancerous Human Breast Epithelium

5. FUNDING NUMBERS
DAMD17-96-1-6026

6. AUTHOR(S)
Terry L. Woodward

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Michigan State University
East Lansing, Michigan 48824-1101

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Extracellular matrix (ECM) proteins are critical for the differentiation of normal mammary epithelial cells, but pronounced changes in the ECM occur during breast cancer. We have determined that estrogen receptor-positive breast cancer cells (MCF-7 and T47D) cultured on laminin have reduced, or lack, estrogen responsiveness. Estrogen-induced proliferation, following anti-estrogen treatment, did not occur on laminin (T47D) or was significantly reduced (MCF-7), but did occur on collagen I or fibronectin. Laminin inhibited basal proliferation of MCF-7, T47D and the ER- negative MDA MB231 cells, but did not inhibit IGF-I or EGF induced proliferation, indicating that tumor cells on laminin do not become refractory to mitogens. An endogenous estrogen-regulated protein in MCF-7 cells, the progesterone receptor, was not significantly stimulated by estrogen on laminin, but was stimulated on collagen I or fibronectin, indicating that laminin may inhibit estrogen receptor binding or post-receptor events. However, estrogen receptor content, in both MCF-7 and T47D cells, was not altered on different ECM proteins. Estrogen stimulation of ERE transcription, from a transfected ERE-luciferase reporter construct, was significantly lower in MCF-7 cells cultured on laminin than on collagen I or fibronectin. Since specific ECM proteins may confer and others inhibit estrogen action in breast cancer cells, therapeutic strategies may be designed to alter the ECM or integrin-ECM binding in the tumor microenvironment to prevent tumor growth and metastasis.

14. SUBJECT TERMS
Breast Cancer
Extracellular Matrix, Estrogen, Progesterone Receptor, Cell Proliferation, Breast Cancer Cells

15. NUMBER OF PAGES
61

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

TW Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Tony Woodward 10/28/98

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

While more than 60% of breast cancers are estrogen-receptor (ER) positive, only one third of estrogen receptor-positive tumors respond to endocrine therapy (Martin et al., 1994). Understanding the mechanisms underlying the acquisition and loss of estrogen responsiveness in breast epithelium may be critical to the treatment of breast cancer since estrogen-responsive tumors have a better prognosis and are less likely to become metastatic than estrogen non-responsive tumors (Garcia et al., 1992; Moghazy et al., 1992). In the normal mammary gland, epithelial-stromal interactions are required for steroid-induced mammary gland growth and morphogenesis. However, breast carcinogenesis is often accompanied by pronounced changes in the stroma, termed desmoplasia, or 'the stromal reaction' (Ronnov-Jessen et al., 1996). In fact, desmoplasia is a prominent feature of infiltrating ductal carcinomas, which are the most common type of breast cancer. Since desmoplastic stroma has marked changes in both cellular composition and secreted proteins, it is likely that normal epithelial-stromal interactions, essential for estrogen-mediated proliferation, are altered during breast tumorigenesis.

Normal mammary epithelial cells in culture do not proliferate in response to ovarian steroids. However, mammary epithelial cells can proliferate in response to estrogen or progesterone when cultured with stromal cells or stromal-derived proteins (McGrath, 1983; Haslam, 1986; Xie and Haslam, 1997). In vivo, the surgical recombination of mature mammary stroma with immature mammary epithelium, transplanted into the mammary fat pad of an immature mouse, permits the precocious development of epithelial estrogen-inducible PR, required for branching morphogenesis (Haslam and Counterman, 1991). To determine if stromal tissue is required for estrogen-mediated mammary gland development, Cunha and colleagues (1997) performed

surgical tissue recombinations of wild-type ($ER^{+/+}$) or ER knockout ($ER^{-/-}$) mammary epithelium with $ER^{+/+}$ or $ER^{-/-}$ mammary stroma and transplanted them under the kidney capsule of athymic nude mice. Mammary gland ductal elongation and morphogenesis occurred when $ER^{+/+}$ stroma was combined with either $ER^{-/-}$ or $ER^{+/+}$ epithelium, but did not occur when $ER^{-/-}$ stroma was combined with either $ER^{-/-}$ or $ER^{+/+}$ epithelium. Collectively, these results demonstrate that normal mammary stromal is required for ovarian steroid mediated morphogenesis and proliferation of normal mammary epithelium.

Two major mechanisms by which stromal cells can influence epithelial cell behavior are by the secretion of growth factors and by altering the molecular composition of the extracellular matrix (ECM). In the normal mammary gland, stromal cells synthesize the major extracellular matrix proteins in the epithelial basement membrane (Keely et al., 1995). Our laboratory has previously demonstrated a requirement of specific ECM proteins, fibronectin (FN) or collagen IV (Col IV), for progesterone-induced proliferation of cultured mouse mammary epithelial cells (Xie and Haslam, 1997). Other ECM proteins are required for differentiation. For example, laminin (LM), a major ECM protein in the basement membrane, has been demonstrated to play a critical role in the lactational differentiation of normal mammary epithelium in vitro (Schmidhauser et al., 1990; Streuli et al., 1991). The effects of ECM proteins on estrogen action in breast cancer cells have not been determined.

In our previous annual report we had found that estrogen-induced cell proliferation in MCF-7 and T47D cells was modified by the attachment to ECM proteins. Specifically, laminin inhibited proliferation of both cell lines, while collagen I, collagen IV, fibronectin and vitronectin

permitted estrogen-induced proliferation. We also determined that estrogen-induction of progesterone receptor in MCF-7 cells occurred on all ECM proteins, but was significantly lower on laminin. These experiments were all performed in the presence of charcoal stripped FBS.

Next, serum-free culture conditions were developed that would permit estrogen-responsiveness to determine the direct effects of isolated ECM proteins, as serum contains hormones, growth factors and ECM proteins. When plated and cultured in serum-free media without growth factors, estrogen did not stimulate proliferation of either cell line. Insulin and growth factors (EGF and IGF-I) have been reported to permit various estrogen-mediated responses in MCF-7 cells and T47D cells, when used at submaximal concentrations. Thus, EGF and IGF-I dose response curves were determined on collagen I for both MCF-7 and T47D cells. EGF and IGF-I significantly stimulated proliferation at concentrations as low as 5 and 25 ng/ml for both cell lines. At these concentrations of growth factors, estrogen stimulated proliferation of MCF-7 cells on collagen I, and T47D cells on collagen I, fibronectin and vitronectin (NOTE: we have repeated some of the serum-free experiments since the first annual report, which was necessary to obtain statistical differences, and determine statistical significance). However, following 4 d of treatment, DNA content of estrogen treated cells was only 20-30% greater than control treated cells on stimulatory ECM proteins for both cell lines.

These serum-free conditions were also used to determine if ECM proteins altered estrogen-induced progesterone receptor in MCF-7 cells. Similar to serum-containing media, E-induced progesterone receptor occurred on all ECM proteins, except laminin. In contrast to proliferation experiments in the absence of serum, we found that estrogen stimulated progesterone receptor

expression to the same extent in the absence of serum as in the presence of serum, when IGF-I and EGF were present. This suggests that these two estrogen-mediated events are dissociated. Our serum-free proliferation assay was insensitive and unlikely to be useful in determining the mechanisms involved in altered estrogen responsiveness on different ECM proteins.

In the current studies, we first needed to develop a more sensitive assay to measure estrogen-induced proliferation in serum-free culture. We used thymidine incorporation and cell cycle staging to determine how estrogen regulates proliferation of the breast cancer cell lines, MCF-7 and T47D and the ER negative MDA MB231 cells on different ECM proteins. Next, we modified the thymidine assays for loosely attached cells, common in cancer cells plated in serum-free media. Cell cycle analysis revealed that a high percentage of MCF-7 cells were in S-phase even in the absence of serum, estrogen and growth factors, and that a pure anti-estrogen (ICI 182,780) blocked proliferation. Pretreatment with ICI 182,780 reduced basal proliferation and greatly enhanced the estrogen-induced thymidine incorporation. The increased sensitivity made it possible to further examine the mechanisms involved in altered estrogen responsiveness on different ECM proteins.

We report that estrogen-induced proliferation and progesterone receptor induction in two ER positive breast cancer cell lines, MCF-7 and T47D, occurs on collagen I (Col I), Col IV, FN and vitronectin (VN). However, estrogen-induced proliferation and PR induction were significantly lower when cells were cultured on LM. Altered estrogen responsiveness of MCF-7 and T47D cells was not due to cellular ER concentrations. Furthermore, culture of cells on LM did not prevent all mitogenic responses, as IGF-I or EGF induced proliferation on LM. Instead, culture

of cells on LM inhibited estrogen-stimulation of estrogen response element (ERE) reporter activity, thereby inhibiting the transcriptional activity of the ER.

These experiments, therefore, will examine the role that ECM proteins have in E-induced events in breast cancer cells, and determine which specific ECM proteins may influence E-action. Identification of either acquisition or loss of E-action by specific ECM proteins, should shape future research in determining how ECM may influence normal mammary growth and tumor growth.

BODY

Experimental Methods:

Materials

Culture media, phenol red-free DMEM/Ham's F12, was obtained from Sigma Chemical Co. (St. Louis, MO). Rat Col I, mouse Col IV, ultrapure-entactin-free mouse LM, and human VN were purchased from Becton/Dickinson Labware (Bedford, MA). Human FN was purchased from ICN Biomedicals, Inc. (Aurora, OH). The antiestrogen ICI 182,780 was a gift from ICI Pharmaceuticals (Macclesfield, Cheshire, England). Radioinert R5020 (promogestone), [17β-Methyl-^{3}H]Promogestone (R5020, 85 Ci/mmol), and Methyl-^{3}H-Estradiol-17β (120 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA). Methyl-^{3}H-thymidine (50 Ci/mmol) was purchased from ICN Biomedicals, Inc. Nonessential amino acids (NEAA), gentamycin, certified FBS, and insulin were obtained from Life Technologies, Inc. (Grand Island, NY). All culture plates were purchased from Corning Laboratories, Inc. (Corning, NY). Charcoal/Dextran treated DCC-FBS was from Hyclone Laboratories, Inc. (Logan, UT). Insulin-like growth factor-I (IGF-I) was purchased from GroPep Pty. Ltd. (Adelaide, Australia). Poly-L-lysine and all other hormones and growth factors were obtained from Sigma Chemical Co. (St.

Louis, MO).

Cell lines, culture conditions, plasmids and ECM coating

MCF-7 and T47D human ER positive, estrogen-responsive breast cancer cells were kindly provided by Dr. W. Helferich, Univ. Illinois, Urbana, IL and Dr. B.K. Vonderhaar, NIH, Bethesda, MD, respectively. ER negative MDA MB231 cells were also provided by Dr. W. Helferich. MCF-10A TG1 and MCF-10A TG3c cells were provided by Dr. M. Shekhar, Karmonos Cancer Inst., Detroit Mich. These lines were derived from MCF-10A spontaneously immortalized normal human breast cells. The T24 ras transfected TG series include the non-transformed TG1 and the transformed TG3c. These cells were cultured in DME/F12 containing 5% FBS, 10 nM estrogen, 125 ng/ml insulin, and 50 ug/ml gentamycin for routine culture. Experimental media contained phenol-red free DME/F12 + 5% DCC and 50 ug/ml gentamycin and was used in all serum-containing experiments. Serum-free experimental media contained phenol-red free DME/F12 with 125 ng/ml insulin, NEAA, gentamycin with or without 5 ng/ml EGF and 25 ng/ml IGF-I, as noted. All cells were incubated in 5% CO_2 at 37 C.

ERE-luciferase (pERE-tk109-luc) and control TK-luciferase (ptk109-luc) were kindly provided by Dr. Barry Gehm, Northwestern University Medical School, Chicago, IL (Gehm et al., 1997). A β-galactosidase plasmid, p6RL, was provided by Dr. Donald Jump, Michigan State University, East Lansing, MI.

Col I, Col IV, LM, FN were coated at 6.25 ug/cm^2, PLL at 12.5 ug/cm^2 and VN at 500 ng/cm^2 as previously described (Xie and Haslam, 1998).

Serum containing DNA assays

MCF-7, T47D or MDA MB 231 cells were plated at 7.5 X 10^3 cells/cm^2 in 24-well dishes for 24 h, switched to experimental media for 2 d, media changed, treatments applied for 4 d and cells harvested for DNA assay. Treatments were changed every 2 d. DNA content was determined by a fluorometric assay using Hoescht 33258 dye (West et al., 1985).

Serum-free DNA assays

MCF-7, T47D or MDA MB 231 cells were plated at 3.75 X 10^4 cells/cm^2 in 24-well dishes in serum-free experiment media for 2d with 5 ng/ml EGF and 25 ng/ml IGF-I, and treatments added for 4 d similar to serum containing DNA assays.

^{3}H-thymidine assays

Cells were assayed for ^{3}H-thymidine incorporation after treatment with the anti-estrogen ICI 182,780 and recovery in estrogen as previously described. Briefly, cells were plated at 6 X 10^4 cells/cm^2 in 24 well dishes in serum-free experiment media with 5 ng/ml EGF and 25 ng/ml IGF-I for 24 h, then 2X10^{-7} M ICI 182,780 added for 2 d. Cells were subsequently rinsed and treatments (2 X 10^{-7} M ICI 182,780, no treatment or 1 X 10^{-8} M estrogen) added for 20 h. One uCi methyl-3H-thymdine/well was added for 2 h and cells transferred to GFC filters (Whatman International, Ltd., London England), the well rinsed 1X with HBSS, and the filter rinsed 2X each with ice cold HBSS, 10% TCA and 90% ethanol. Filters were transferred to liquid scintillation vials and radioactivity determined. ^{3}H-thymidine incorporation per well was adjusted by DNA content per well.

Steroid hormone binding assay

Ligand binding assay were used to determine estrogen receptor and progesterone receptor content as previously described (Xie and Haslam, 1998). Briefly, for ER binding assays, cells were cultured in 24-well plates in serum-free treatment media with EGF (5 ng/ml) and IGF-I (25 ng/ml) for 3 days, incubated with 14 nM ^{3}H-estradiol with or without 250-excess unlabeled estradiol (3.5 μM) for 1 h at 37 C. Cells were transferred to GFC filters and rinsed 3 times with HBSS. Filters were transferred to liquid scintillation vials and radioactivity determined. PR content was determined after 3 d estrogen or control treatment. 8 nM radiolabeled R5020 plus 500-fold excess dexamethosone (total binding) or 500-fold excess unlabeled R5020 (non-specific) were added for 1 h at 37 C. Dexamethosone was added to suppress R5020 binding to glucocorticoid binding sites. ER and PR per well were adjusted by DNA content per well.

ERE luciferase, protein and β-galactosidase assays

Cells were plated at 1.2 X $10^5/cm^2$ in 6-well dishes in serum-free treatment media with EGF (5 ng/ml) and IGF-I (25 ng/ml) for 24 h, transfected with 2 ug/well ERE-luciferase or TK-luciferase or 1.5 ug/well p6RL (β-galactosidase plasmid) using Superfect transfection reagent (Qiagen, Inc., Valencia, CA) for 2 hours. Treatments, estrogen (10 nM), no treatment, or ICI 182,780 (200 nM), were added directly following transfection for 24 h, and cells lysed for luciferase, protein and β-galactosidase assays in luciferase reporter lysis buffer.

Luciferase activity was determined using a Promega Luciferase Assay System with Reporter Lysis Buffer (Promega Corp., Madison, WI), as per Promega Technical Bulletin 161 and read on a Turner TD-20e luminometer (Turner Designs, Inc.).

Protein content was determined by the Bio-Rad Protein microtiter plate assay (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm.

B-galatosidase content was determined using Chlorophenol Red-β-D-galactopyranoside sodium salt (CPRG, Boehringer Mannheim, Indianapolis, IN) and read at 575 nM, B-galactosidase was used as a standard.

Statistical analysis

All data were expressed as the mean ± SEM. Differences between means were tested for statistical significance using the Student t-test or paired Student t-test where appropriate.

Results

Estrogen-induced proliferation on ECM proteins in the presence of serum

Two estrogen receptor (ER) positive, MCF-7 and T47D, and one ER negative cell lines, MDA MB231, were used to study the effects of ECM proteins on estrogen-responsiveness. In the presence of 5% charcoal stripped (DCC) fetal calf serum, attachment of all cell lines was greater

than 90% on all ECM proteins (data not shown). In the absence of estrogen, DNA/well of MCF-7, T47D or MDA MB231 cells was similar on plastic, Col I, Col IV, FN and VN, but DNA/well was lower when cultured on LM for 4 d, and significantly lower for both T47D (except plastic) and MDA MB231 cells ($P<.05$) (Figure 1A,B,C).

Estrogen treatment for 4 d significantly ($P<.05$) increased DNA/well of MCF-7 and T47D cells by 1.6 to 1.9-fold on Col I, Col IV, FN or VN (estrogen treatment did not significantly increase DNA content of MCF-7 cells plated on VN, $P<.075$) (Figure 1A,B). MCF-7 and T47D cells plated on LM, were not significantly growth stimulated by estrogen (Figure 1A,B). MBA MD231 cells did not show a proliferative response to estrogen on any ECM (Figure 1C). These results demonstrate that in the presence of serum, estrogen-induced growth of ER positive breast cancer cells is altered by ECM proteins. However, since serum contains ECM proteins and other attachment factors, such as FN and VN, we wished to investigate the distinct effects of specific ECM proteins, under hormone and growth factor defined conditions. To do this, we developed the minimal serum-free culture conditions that support estrogen responsiveness of the MCF-7 and T47D cells.

Estrogen-induced proliferation on ECM proteins in the absence of serum

Others have found substantially reduced responses to estrogen in the absence of IGF-I and/or EGF in MCF-7 and T47D cells in serum-free media (Page et al., 1983; Van Der Burg et al., 1988; Katzenellenbogen and Norman, 1990). Similarly, we found that in the absence of growth factors, neither T47D nor MCF-7 cells showed a proliferative response to estrogen when plated in serum-free media. Thus, dose response studies measuring DNA content after 4 days culture in growth factor containing media were performed to determine the minimum concentration of

IGF-I and/or EGF that were necessary to obtain an estrogen-response when cells were plated and cultured in serum-free media. The minimum concentrations of EGF and IGF-I required for estrogen stimulation of proliferation were 5 ng/ml and 25 ng/ml, respectively on Col I. At these concentrations of growth factors, estrogen increased DNA content of both MCF-7 and T47D cells on Col I (Figure 2 A,B). Next, proliferation of MCF-7 and T47D cells by estrogen in the presence of IGF-I and EGF on all ECM proteins was determined. As before, estrogen stimulated MCF-7 cell proliferation 20-30% on Col I, but not on any other ECM protein (Figure 3A). In T47D cells, estrogen significantly increased proliferation on Col I, FN and VN (Figure 3B). Estrogen did not stimulate proliferation of MDA MB231 cells on any ECM protein (Figure 3C). These results demonstrate that EGF and IGF-I enhanced estrogen-induced proliferation of MCF-7 and T47D cells on specific ECM proteins, suggesting that growth factor, steroid and ECM pathways converge to mediate cell proliferation.

To determine if LM inhibited all proliferative responses, dose response studies of MCF-7 cells (and are currently being done for T47D cells) on Col I, LM, and FN proteins were performed, using thymidine incorporation. IGF-I stimulated thymidine incorporation at similar concentrations on all ECMs, with a maximal increase of 2.3-3-fold on all ECMs (data not shown). Stimulation of proliferation by EGF also occurred at similar concentrations and induced similar responses on all ECMs. Before addition of growth factors, ^{3}H-thymdine incorporation was slightly lower than fibronectin and collagen I. However, both EGF and IGF-I were effective at increasing proliferation similarly on all ECMs. These data suggest that culture of MCF-7 cells on LM does not prevent the cells from responding to all mitogens, by leaving the cell cycle and differentiating. This is in agreement with other data that determined that the percentage of MCF-

7 cells in S-phase on Col I and LM in the presence of stripped serum was not different.

Cell cycle staging, by propidium iodine staining and flow cytometric analysis, of MCF-7 cells in estrogen-free, serum-free, growth factor-free media revealed that as high as 30% of cells were in S-phase. The anti-estrogen ICI 182,780 significantly reduced the percentage of S-phase cells to 10% (data not shown), indicating that ER may still be activated in these estrogen-starved and serum-starved cells. The activation of ER, by residual estrogen or more likely by growth factor pathways (Ignar-Trowbridge et al., 1996), have masked estrogen-induced proliferation in serum-free media. To increase the sensitivity of our proliferation assays, cells were treated with the anti-estrogen ICI 182,780 and ^{3}H-thymidine incorporation measured after treatment with estrogen as previously described (Weinberg et al., 1997). Thymidine incorporation into DNA was 7-10-fold higher in MCF-7 cells treated with estrogen on Col I or FN than in control treated cells (Figure 4A). Strikingly, MCF-7 cells plated on LM had a significantly smaller 2-fold increase in thymidine incorporation upon estrogen treatment, though this estrogen-induced increase in proliferation was significant.

After ICI 182,780 pretreatment of T47D cells, estrogen increased thymidine incorporation 1.5 to 2-fold on FN or Col I, respectively (Figure 4B). Thymidine incorporation in T47D cells was not stimulated by estrogen on LM. Since T47D cells are only weakly adherent to LM in the absence of serum, we wished to determine whether the strength of attachment to LM contributed to the lack of estrogen-responsiveness. In the presence of serum, the percentage of T47D cells attached to ECM proteins is similar to serum-free culture, but the strength of attachment is greater as evidenced by cell detachment during multiple rinsing steps. We repeated these

experiment in serum-containing media. Again, estrogen treatment significantly increased thymidine incorporation on Col I and FN, but not on LM (Figure 4C). Therefore, the strength of adhesion was not a determinant of the lack of estrogen-induced proliferation on LM. MDA MB231 cells treated with ICI, did not exhibit estrogen-inducible proliferation on any ECM protein examined (Figure 4D).

Estrogen receptor status

Since both MCF-7 and T47D cells plated on LM showed little or no proliferation in response to estrogen, we hypothesized that ER levels may be down-regulated when cells were plated on LM. However, MCF-7 and T47D cells had similar levels of ER regardless of ECM (Figure 5). ER content, as determined by ligand binding, was lower in T47D cells than in MCF-7 cells as has been previously reported (Ruedl et al., 1990). These results indicate that the decrease in estrogen responsiveness of MCF-7 and T47D cells cultured on LM was not due to a decrease in ER concentration or ER binding, since culture of cells on different ECM proteins did not alter radiolabeled estrogen binding to the ER. MDA MB231 cells were ER negative on all ECM proteins.

Induction of estrogen regulated progesterone receptor

MCF-7 cells contain estrogen inducible progesterone receptors. Since estrogen-induced proliferation was altered in MCF-7 cells it was of interest to determine if estrogen regulation of PR was altered by ECM. T47D cells were not used in these experiments since they have high levels of PR that are not regulated by estrogen (Horwitz et al., 1982). Estrogen increased PR levels on all ECM proteins except LM (Figure 6). In contrast to estrogen-induced proliferation,

estrogen was able to induce PR in the absence of serum to the same extent as in presence of serum (data for serum-containing experiment not shown). Thus, two estrogen-dependent events were down-regulated or lost when MCF-7 cells were cultured on LM.

Activation of ERE-luciferase and AP-1-CAT by estrogen on ECM proteins

The above experiments indicate that estrogen-induced proliferation and PR induction are reduced or absent when MCF-7 cells or T47D cells are cultured on LM. Since lack of response to estrogen on LM was not due to a reduced ER content or binding, we considered the possibility of altered ER function on LM. To test this, we transfected cells with an ERE-luciferase reporter construct, plated cells on different ECMs and analyzed estrogen induction of ERE activity. Loss of activity or significant differences in activity would indicate that ECM proteins might alter cofactors or repressors that permit ER binding to DNA and/or activation of ERE. MCF-7 cells plated on FN and Col I increased ERE-luciferase activity 7 to 10-fold when treated with estrogen, while cells plated on LM had significantly lower induction of ERE-luciferase (Figure 7). As previously reported, T47D cells showed very weak (often non-significant) stimulation of ERE activity, even when co-transfected with estrogen-receptor, preventing accurate testing on various ECM proteins. AP-1 CAT experiments in progress.

Non-transformed human breast cell response to estrogen on ECM proteins

The MCF-10A TG series has previously been reported to be ER positive and respond to estrogen when plated in 3-D soft agar gels (Dr. M. Shekhar, personal communication). These cells have not been found to proliferate in response to estrogen on tissue culture plastic. Additionally, these cells may have a PR splice variant, as Western blot analysis for PR revealed a strong band below

the w.t. molecular weight. Estrogen-responsiveness is rapidly lost in normal mammary epithelial cells in culture. So, this stable cell line series was an excellent opportunity to examine the regulation of estrogen responsiveness in normal cells and as they become transformed. We examined proliferation of both lines in 5% DCC FBS, using DNA and thymidine assays on PLL, Col I, LM and FN. Estrogen did not stimulate thymidine incorporation with short- (18-24 h treatment) or longer (42-48 h) treatment in either the MCF-10A TG1 or TG3c cells on any ECM protein. In the absence of estrogen, thymidine incorporation was lowest for both the TG1 and TG3c cells on LM at 24 h (Figure 8), but by 48 h proliferation was similar on all ECM proteins (Figure 9). DNA content of TG1 and TG3c was not stimulated by estrogen on any ECM protein, either (Figure 10). Initially, we found specific ^{3}H-R5020 binding, in the presence of 250-fold excess cold R5020. The binding of R5020 was not altered significantly by either E or culture on different ECM proteins (Figure 11). This implies that there are specific PR, that are not regulated by estrogen or ECM. We compared PR immunocytochemical staining in the TG1 and TG3c to staining in the MCF-7 and T47D cells. MCF-7 cells had very clear staining, while the T47D cells had approximately 10-fold greater staining intensity (this corresponds to the previous results we have gotten with ligand binding experiments and with published results). Alternatively, no staining was present in the TG1 or TG3c cells. A scathard analysis for PR revealed no PR, only low affinity binding that could not be competed out with unlabeled progesterone. Therefore, the TG1 and TG3c series, in monolayer culture, do not proliferate in response to estrogen on any ECM and do not have PR, so we did not continue experiments with these cell lines.

Discussion

This study demonstrates that specific ECM proteins can differentially regulate estrogen-mediated events in breast cancer cells. We have previously found that normal primary mouse mammary epithelial cells from nulliparous animals proliferate in response to progestin (R5020) on FN and Col IV, but not on PLL, Col I, tenascin, or LM (Xie and Haslam, 1997). We hypothesized that a similar mechanism may exist for estrogen-induced proliferation of breast cancer cells in culture; such that certain ECM proteins may be required for estrogen responsiveness. Instead, MCF-7 and T47D breast cancer cells proliferated on most ECM proteins examined, including Col I, Col IV, FN and VN, in response to estrogen when cells were cultured in the presence of growth factors (IGF-I and EGF) or serum. However, in the presence or absence of serum, breast cancer cells on LM exhibited little to no estrogen-induced proliferation. Furthermore, estrogen did not significantly increase progesterone receptors in MCF-7 cells on LM. Estrogen receptor content was not altered when cells were cultured on different ECM proteins. However, estrogen was significantly less effective in stimulating ERE activity when MCF-7 cells were cultured on LM, indicating LM specifically inhibits estrogen-mediated transcription of genes containing ERE. These data support the role of the tumor extracellular microenvironment in influencing hormonal responsiveness, critical to tumor growth and breast cancer therapies.

ECM regulated proliferation has not been well studied in the breast. However, LM induction of a differentiated phenotype in cultured normal mammary epithelial cells, including expression of milk proteins and morphological differentiation, have been well studied. ECM proteins have also been clearly demonstrated to alter invasion and haptotaxis of cultured breast cancer cells. ECM proteins regulate cell morphology of breast cancer cells, as culture of MCF-7 cells on LM

coated dishes or addition of soluble LM to cells plated on plastic caused cells to form clusters and prevented spreading and migration. Whereas, MCF-7 cells on FN had a flattened morphology and increased cell spreading (Noel et al., 1988; Coopman et al., 1991). Similarly, we have found an increase in rounding and clustering of MCF-7 and T47D cells on LM. Alternatively, LM did not alter the flattened morphology of the ER-negative MDA MB231 cells. Regulation of normal and breast cancer cell proliferation by ECM proteins has also been reported (Pourreau-Schneider et al., 1984; Elliott et al., 1992; Xie and Haslam, 1997). We have found that normal mammary epithelial cells from nulliparous mice in serum-free culture proliferate in response to progesterone on Col IV or FN, but not on Col I, LM or tenascin (Xie and Haslam, 1997). Using less defined ECM preparations, Pourreau-Schneider and coworkers (1984) examined estrogen responsiveness of MCF-7 breast cancer cells. Little difference in growth responsiveness to estrogen was found when MCF-7 cells were plated on plastic, a crude corneal endothelial cell ECM preparation, or Col I in the presence of serum. This study did find that culture of MCF-7 cells in 3-D spheroid suspension culture enhanced estrogen-induction of PR. This enhanced response to estrogen may have been mediated by secreted ECM proteins, however, since other mammary carcinoma cell lines have been reported to increase secretion of FN when cultured as spheroid colonies in suspension (Saulnier et al., 1996). The present study is the first to demonstrate regulation of estrogen regulated events in breast cancer cells by specific purified extracellular matrix proteins in serum-free conditions.

ECM proteins interact with growth factors to regulate proliferation of many cell types (Elliott et al., 1992; Bohmer et al., 1996; Wary et al., 1996). ECM proteins bind to cell surface integrins and can activate focal adhesion kinase and several intracellular kinases that are also regulated by

growth factors, including MAP kinases, protein kinase C, and PI-3 kinase (Bohmer et al., 1996). ECM-integrin binding can enhance, alter or inhibit growth factor mediated responses (Wary et al., 1996). This report and others have demonstrated that IGF-I and EGF may be necessary for normal estrogen action and in some circumstances activate estrogen receptor pathways independent of estrogen. Insulin or IGF-I have been reported to be required for estrogen induction of PR (Katzenellenbogen and Norma, 1990), while Van de Burg and colleagues (1988) found that MCF-7 cells require insulin or IGF-I to proliferate in response to estrogen. Both studies used serum containing media to plate cells, however. We have determined that MCF-7 and T47D cells plated in serum-free media without growth factors are not growth stimulated by estrogen, but in the presence of IGF-I and EGF, both cells can respond to estrogen on the appropriate ECM protein. Furthermore, IGF-I and EGF increased ERE-luciferase expression 1.5-2-fold in the absence or presence of estrogen (after adjusting for transfection efficiency) in MCF-7 cells on Col I (data not shown). This was not unexpected, as EGF has been demonstrated to activate the unliganded ER through the MAP kinase pathway (Bunone et al., 1996). Also, EGF, TGF-alpha and IGF-I have been shown to enhance transcription of an ERE-CAT construct independent of estrogen, but this was blocked by the anti-estrogen ICI 164,384 (Ignar-Trowbridge et al., 1996). Again, these cells were plated or cultured in serum-containing media, so it was not clear what effect ECM proteins had on the regulation of estrogen-induced proliferation. These results demonstrate that IGF-I and EGF may activate ER independent of estrogen or synergize with estrogen to stimulate ERE transcriptional activity, but only when cultured on specific ECM proteins. In agreement, Wary and colleagues (1996) have demonstrated that different classes of integrins may either cooperate with mitogens, promoting cellular proliferation, or preventing mitogen action, demonstrating that attachment to an

appropriate substrata acts as a master regulator of cell function.

We have determined that culture of breast cancer cells on LM can inhibit estrogen action even if appropriate growth factors are present. However, unlike our studies, many non-transformed cells can completely shift from a differentiation to proliferation responsive state or vice-versa by culture on specific classes of ECM proteins or by ligation of specific integrins. For example, LM has been reported to induce differentiation or apoptosis and prevent proliferation of many normal cells, while FN or VN block differentiation and induce or potentiate proliferation. Wary and colleagues (1996) found that ligation of FN ($\alpha 5$) or VN ($\alpha v \beta 3$) receptors recruit Shc-Grb2 which activates the MAP kinase pathway and cooperates with mitogens to promote transcription from the Fos-serum response element to permit cell cycle progression. Laminin and the $\alpha 6$-LM receptor did not activate this pathway, but instead induced apoptosis. Other researchers have found similar results. For instance, Sastry and colleagues (1996) found that over-expression of alpha 5 (FN receptor) or antisense knockout of alpha 6 (LM receptors) resulted in decreased differentiation of muscles cells and increased proliferation, while differentiation was correlated with enhanced alpha 6 expression and reduced alpha 5 expression. Endothelial cells have been reported to proliferate on FN coated plates, but on a LM-rich extracellular matrix they stop growing and form capillary-like structures (Kubota et al., 1988). We have also found that LM, but not Col I or FN, generally decreased basal cell proliferation in the absence of estrogen of MCF7, T47D and MDA MB231 cells, and significantly decreases estrogen-induced proliferation. However, dose response studies of MCF-7 and T47D cells for IGF-I or EGF on different ECMs revealed that thymidine incorporation occurred at similar growth factor concentrations and at similar rates on different ECM. These data demonstrate that culture of

breast cancer cells on LM does not eliminate all mitogenic responses, indicating that inhibition of estrogen mediated events does not occur solely because cells are moved from a proliferative to a differentiation state.

Alternatively, lack of estrogen responsiveness may have occurred on LM because attachment was weaker on LM. Giancotti and Mainiero (1994) have presented a convincing hypothesis that growth of cells is maximal when moderate adhesion to extracellular matrix proteins occurs, while strong adhesion or lack of adhesion are not permissive to growth. When either T47D cells or MCF-7 cells were plated on LM, the cells appeared less flattened, and were more easily removed during rinsing. Consequently cells cultured on LM may have had a looser adherence to the substratum. Loss of estrogen responsiveness on LM cannot be solely attributed to attachment or strength of attachment, as other ECM proteins that promoted similar attachment under serum-free conditions, such as FN, did not inhibit estrogen responsiveness, and the stronger attachment of T47D cells in the presence of serum to LM did not permit estrogen-mediated proliferation (Figure 4B vs. 4C).

Our data demonstrated that culture of MCF-7 cells on LM interfered with estrogen receptor mediated transcription pathways. Laminin does not directly regulate ER levels though, since breast cancer cells plated on different ECM proteins did not have different ER concentrations, as determined by ligand binding assays. This indicates that ER concentration, and ER binding does not explain altered estrogen function when cells were plated on LM. The decrease in ERE-luciferase activity corresponded well with the decrease in estrogen stimulation of thymidine incorporation in MCF-7 cells pretreated with the anti-estrogen ICI 182,780. Laminin was able to

significantly stimulate both proliferation and ERE-luciferase activity, but in both cases the stimulation was significantly lower than cells cultured on FN or Col I. Since PR is a endogenous estrogen regulated protein in MCF-7 cells, we examined regulation of PR induction. Similarly, we found that MCF-7 cells on LM had a small estrogen-induced increase in PR (not significant), while cells on all other ECM proteins had a larger significant estrogen-induction of PR. This indicates that regulation of ER mediated transcription is a major mechanism by which LM influences estrogen action. We did not examine other estrogen-regulated genes. It is possible that not all estrogen regulated genes are inhibited by LM, as others have shown a dissociation between regulation of different estrogen mediated genes (Huey et al., 1998). Inhibition of steroid coactivators or over-expression of corepressors are probable candidates for the reduced activation of ERE transcriptional activity on LM.

The regulation of estrogen action in breast cancer cells may be particularly relevant to advanced breast cancer. Breast carcinogenesis is frequently accompanied by desmoplasia, pronounced changes in stromal tissue, accounting for as much as 90% of the tumor (Dvorak, 1986) . Desmoplastic stroma have altered synthesis and secretion of growth factors and ECM proteins. Misregulated expression and over-expression of ECM proteins including collagens, elastin, FN, LM, tenascin, proteoglycans and glycosaminoglycans have been documented (Ronnov-Jessen et al., 1996). The altered ECM in tumors may increase the metastatic potential of tumor cells and prevent immune responses to the tumor or inhibit tumor cell growth and invasion, depending on the ECM produced (Ronnov-Jessen et al., 1996). Additionally, LM and LM receptors are often expressed by the malignant breast cancers cells as well. Interestingly, these malignant cells are less likely to be responsive to estrogen than non-malignant or normal cells. Our results which

have determined that collagens and FN permit, while LM inhibits, estrogen action in breast tumor cells indicate that the altered pattern of ECM expression during progression of breast cancer may alter estrogen responsiveness and growth of the tumor.

This study demonstrates for the first time that extracellular matrix proteins influence estrogen action in breast cancer epithelial cells. Since stromal cells are the major source of epithelial basement membrane proteins in the normal gland (Keely et al., 1996) and this pattern is dramatically altered during carcinogenesis, expression of different extracellular matrix proteins may be a major pathway by which stromal cells influence epithelial cell behavior. This study demonstrates that the tumor microenvironment influences epithelial cell responsiveness to hormones and may lead to hormone insensitivity. The loss of hormone responsiveness in breast cancer is associated with a poor prognosis and substantially limits treatment options. Advancing our understanding of the mechanism(s) by which ECM proteins influence hormone responsiveness and tumor growth may lead to novel therapeutic strategies for the treatment of breast cancer.

Recommendations to Statement of Work

We have determined that laminin inhibits estrogen action in breast cancer cells. Originally, we had hypothesized that certain ECM proteins may potentiate the effects of estrogen, similar to what we had found for progesterone in normal mammary epithelial cells (that progesterone only stimulates proliferation maximally on FN). Instead, estrogen-induced proliferation, induction of progesterone receptor and induction of ERE transcription occurred on all ECM proteins, but was induction was smaller or did not occur on laminin. These unexpected results have caused us to

reformulate several aspects of our statement of work. We have painstakingly developed highly sensitive assays to measure estrogen responsiveness in these cell lines, and directly measured estrogen receptor activity. Recently, estrogen binding to either ERα or ERβ has been shown to signal through an AP1 site, estrogen binding to ERα activating transcription and ERβ inhibiting transcription, while antiestrogen binding to ERβ may stimulate transcription (Paech et al., 1997). Since 1) Fos-Jun binding to the AP1 site is involved in cell cycle progression, and 2) laminin may cause exit from the cell cycle in normal mammary epithelial cells and 3) we have shown laminin to inhibit various estrogen-mediated events including ERE transcriptional activity, we are in the process of testing an AP-1-CAT construct on different ECM proteins. If laminin does not alter AP-1 mediated transcription, this would mean that only ERE promoter containing genes are effected. However, if laminin did alter AP-1 transcription, laminin would interfere with multiple ER pathways, perhaps by up-regulation of a shared corepressor or down-regulation of a coactivator.

Progesterone induced cellular proliferation

We originally wanted to examine proliferation of breast cancer cells by estrogen and progesterone. However, recent studies have found that progesterone generally inhibits proliferation of MCF-7 and T47D cells. This inhibition occurs when cells are stimulated with growth factors or serum (Cappelletti et al.,1995). Some reports have found that progesterone may stimulate proliferation, though. For example, in T47D cells, progesterone accelerates cells through the first mitotic cycle, but arrests them in late G1 of the second cycle (Groshong et al., 1997). Furthermore, the cell cycle arrest can not be overcome by subsequent treatments of progesterone, but may enhance responsiveness to growth factors. The complex interactions

necessary for progesterone stimulation of proliferation are not clearly defined, even in the presence of serum. Currently, we have found a novel regulation of estrogen responsiveness by laminin, a protein important in normal lactation as well as metastasis. Since progesterone action is less clearly defined in vivo and in vitro, we are committed to finishing the necessary experiments regarding estrogen responsiveness that will allow for publication of manuscripts before performing progesterone experiments.

Determine which integrins are involved for E-responsiveness or lack of E-responsiveness. Additionally, we are interested in determining which integrin(s) are responsible for signal transduction. In the past year, we have obtained and tested function blocking antibodies for fibronectin ($\alpha5\beta1$) integrins. We will obtain function blocking antibodies to laminin receptors ($\alpha6\beta4$ or $\alpha6\beta1$). We have also received a plasmid containing the cDNA to $\alpha5$ integrin subunit and have requested a plasmid containing the $\alpha6$ subunit. These plasmids may be used to over-express or knockout the integrins (anti-sense) if interesting results are obtained from function blocking antibody experiments. The antibodies will be used to determine if 1) laminin inhibits E-function by specific integrin binding, and 2) if fibronectin influences E-function by its classical integrin ($\alpha5\beta1$). It would also be of interest to determine the effects of blocking fibronectin binding to the $\alpha5\beta1$ in the presence of serum on T47D and MCF-7 cell responsiveness to E, since a major attachment factor in serum is fibronectin.

Additionally, we have obtained antibodies for immunocytochemistry that we have tested in the past year for fibronectin, collagen I, collagen IV, laminin, $\alpha6$ integrin subunit, αv integrin subunit and we are currently testing several $\alpha5$ antibodies. These experiments are included in Specific Aim #3 of the grant, but have been modified with antibodies and cDNAs to specific

integrin subunits that appear to be important in estrogen-mediated signaling, or inhibition thereof according to results we have obtained.

CONCLUSIONS

This study demonstrated that specific ECM proteins can differentially regulate estrogen-mediated events in breast cancer cells. MCF-7 and T47D breast cancer cells proliferated on most ECM proteins examined, including Col I, Col IV, FN and VN, in response to estrogen when cells were cultured in the presence of growth factors (IGF-I and EGF) or serum. However, in the presence or absence of serum, breast cancer cells on LM exhibited little to no estrogen-induced proliferation. Pretreatment of MCF-7 or T47D cells with the anti-estrogen, ICI 182,780, followed by estrogen treatment confirmed our previous serum-free results, that estrogen is a strong mitogen when cells are plated on collagen I or fibronectin, but little to no estrogen-induced proliferation occurred on laminin. Furthermore, estrogen did not significantly increase progesterone receptors in MCF-7 cells on LM. T47D cells have constitutively high expression of progesterone receptors, that are not estrogen regulatable. Estrogen receptor content was not altered when cells were cultured on different ECM proteins. However, estrogen was significantly less effective in stimulating ERE activity when MCF-7 cells were cultured on LM, indicating LM specifically inhibits estrogen-mediated transcription of genes containing ERE. Subsequent experiments will examine regulation of AP1 transcription by estrogen and anti-estrogens on different ECM proteins. Stromal cells surrounding normal and tumor breast tissue will be analyzed for ECM protein expression and the effect of the secreted ECM proteins on estrogen-induced breast cancer cell proliferation will be measured. These data support the role of the tumor extracellular microenvironment in influencing hormonal responsiveness, critical to tumor growth and breast cancer therapies.

REFERENCES

Bohmer, R-M., E. Scharf, and R.K. Assoian. (1996). Cytoskeletal Integrity is required throughout the mitogen stimulation phase of the cell cycle and mediates the anchorage-dependent expression of cyclin D1. *Molec. Biol. Cell*, **7**:101-111.

Bunone, G., P.A. Briand, R.J. Miksicek, and D. Picard. (1996). Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.*, **15**:2174-2183.

Cappelletti, V., P. Miodini, L. Fioravanti, and G. Di-Fronzo. (1995). Effect of progestin treatment on estradiol- and growth factor-stimulated breast cancer cell lines. *Anticancer Res.*, **15**:2551-2555.

Coopman, P., B. Verhasselt, M. Bracke, G. De Bruyne, V. Castronovo, M. Sobel, J.M. Foidart, F. Van Roy, and M. Mareel. (1991). Arrest of MCF-7 cell migration by laminin in vitro: possible mechanisms. *Clin. Exp. Metastasis*, **9**:469-484.

Cunha, G.R., P. Young, Y.K. Hom, P.S. Cooke, J.A. Taylor, and D.B. Lubahn. (1997). Elucidation of a role for stromal steroid receptors in the mammary gland growth and development using tissue recombination experiments. *J. Mammary Gland Biol. Neoplasia, 2*:393-402.

Elliott, B., A. Ostman, B. Westermark, and K. Rubin. (1992). Modulation of growth factor responsiveness of murine mammary carcinoma cells by cell matrix interactions: correlation of cell proliferation and spreading. *J. Cell Physiol.* **152**:292-301.

Garcia, M., D. Derocq, G. Freiss, and H. Rochefort. (1992). Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of these cells. *Proc. Natl. Acad. Sci.* USA, **89**:11538-11542.

Gehm, B.D., J.M. McAndrews, P-Y. Chien, and J.L. Jameson. (1997). Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **94**:14138-14143.

Giancotti, F.G., and F. Mainiero. (1994). Integrin-mediated adhesion and signaling in tumorigenesis. *Biochem. Biophys. Acta* **1198**:47-64.

Groshong, S.D., G.I. Owen, B. Grimison, I.E. Schauer, M.C. Todd, T.A. Langan, R.A. Sclafani, C.A. Lange, and K.B. Horwitz. (1997). Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27 (Kip1). *Molec. Endocr.*, **11**:1593-1607.

Haslam, S.Z. (1986). Mammary fibroblast influence on normal mouse mammary epithelial cell responses to estrogen in vitro. *Cancer Res.*, **46**:310-316.

Haslam, S.Z., and L.J. Counterman. (1991). Mammary stroma modulates hormonal responsiveness of mammary epithelium in vivo in the mouse. *Endocrinology*, **129**:2017-2023.

Ignar-Trowbridge, D.M., M. Pimentel, M.G. Parker, J.A. McLachlan, and K.S. Korach. (1996). Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. *Endocrinology*, **137**:1735-1744.

Katzenellenbogen, B.S., and M.J. Norman. (1990). Multihormonal regulation of the progesterone receptor in MCF-7 human breast cancer cells: interrelationships among insulin/insulin-like growth factor 1, serum, and estrogen. *Endocrinology*, **126**:891-898.

Keely, P.J., J.E. Wu, and S.A. Santoro. (1995). The spatial and temporal expression of the $\alpha_2\beta_1$ integrin and its ligands, collagen I, collagen IV, and laminin, suggests important roles in mouse mammary morphogenesis. *Differentiation,* **59**:1-13.

Kubota, W., H.K. Kleinman, G.R. Martin, and T.J. Lawley. (1988). Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.,* **107**:1589-1598.

Martin, M.B., M. Saceda, Garcia-Morales, and M.M. Gottardis. (1994). Regulation of estrogen receptor expression. *Breast Cancer Res. Trmt.,* **31**:183-189.

McGrath, C.M. (1983). Augmentation of the response of normal mammary epithelial cells to estradiol by mammary stroma. *Cancer Res.,* **43**:1355-1360.

Moghazy, T., D. Sgoutas, and C. Cohen. (1992).Estrogen and progesterone receptors in breast carcinoma. *J. Med. Res. Inst.,* **13**:165-180.

Noel, A., N. Simon, J. Raus, and J.M. Foidart. (1992). Basement membrane components (Matrigel) promote the tumorgenicity of human breast adenocarcinoma MCF7 cells and provide an in vivo model to assess the responsivenss of cells to estrogen. *Biochem. Pharmacology*, **43**:1263-1267.

Paech, K., P. Webb, G.G. Kuiper, S. Nilsson, J. Gustafsson, P.J. Kushner, and T.S. Scanlan. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science*, **277**:1508-1510.

Planas-Silva, M.D., and R.A. Weinberg, (1997). Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. *Molec. Cell. Biol.*, **17**:4059-4069.

Pourreau-Schneider, N., Y. Berthoois, H. Mittre, C. Charpin, J. Jacquemier, and P.M. Martin. (1984). Estrogen response of MCF-7 cells grown on diverse substrates and in suspension culture: promotion of morphological heterogeneity, modulation of progestin receptor induction; cell-substrate interactions on collagen gels. *J. Steroid Biochem.*, **21**:763-771.

Saulnier, R., B. Bhardwaj, J. Klassen, D. Leopold, N. Rahimi, E. Tremblay, D. Mosher, and B. Elliott. (1996). Fibronectin fibrils and growth factors stimulate anchorage independent growth of a murine mammary carcinoma. *Exp. Cell Res.*, **222**:360-369.

Sastry, S.K., M. Lakonishok, D.A. Thomas, J. Muschler, and A.F. Horwitz. (1996). Integrin α subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation. *J. Cell Biol.*, **133**:169-184.

Schmidhauser, C., M.J. Bissell, C.A. Myers, and G.F. Casperson. (1990). Extracellular matrix and hormones transcriptionally regulate bovine β-casein 5' sequences in stably transfected mouse mammary cells. *Proc. Natl. Acad. Sci. USA,* **87**:9118-9122.

Shi, Y.E., J. Torri, L. Yieh, M.E. Sobel, Y. Yamada, M.E. Lippman, R.B. Dickson, and E.W. Thompson. (1993). Expression of 67 kDa laminin receptor in human breast cancer cells: regulation by progestins. *Clin. Exp. Metastasis*, **11**:251-261.

Streuli, C.H., N. Bailey, and M.J. Bissell. (1991). Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. *J. Cell Biol.,* **115**:1383-1395.

Streuli, C.H., and G. Edwards. (1998). Control of normal mammary epithelial phenotype by integrins. *J. Mam. Gland Biol. Neoplasia*, **3**:151-164.

Tremblay, L., W. Haack, L.T. Nguyen, P. Allard, F. Landry, A. Chapdelaine, and S. Chevalier. (1996). Regulation and activation of focal adhesion kinase and paxillin during the adhesion, proliferation and differentiation of prostatic epithelial cells in vitro and in vivo. *Molec. Endocr.,* **10**:1010-1020.

Van der Burg, B., G.R. Rutteman, M.A. Blankenstein, S.W. de Laat, and E.J.J. van Zoelen. (1988). Mitogenic stimulation of human breast cancer cells in a growth factor-defined medium: synergistic action of insulin and estrogen. *J. Cell. Physiol.*, **134**:101-108.

Woodward, T.L., J.W. Xie, and S.Z. Haslam. (1998). The role of mammary stroma in modulating the proliferative response to ovarian hormones in the normal mammary gland. *J. Mammary Gland Biol. Neoplasia,* **3**:117-131.

FIGURE LEGENDS

Figure 1

Effect of estrogen on proliferation of breast cancer cells on different ECM proteins. DNA content of estrogen receptor-positive, MCF-7 (A), T47D (B), and negative, MDA MB231 (C), cells following 4 d culture in estrogen containing media in the presence of 5% charcoal stripped fetal bovine serum. White bars=control treatment, black bars=10 nM estrogen. Cells were plated on plastic (Plas), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Each value represents the mean ± SEM of 3-8 experiments in Panels A and B. Panel C is data from a representative experiment with error bars of replicates within experiment. *, $P<.05$ that estrogen treated groups have a higher DNA content than control groups. **, $P<.05$ that LM control groups have a lower DNA content than control groups on any other ECM protein.

Figure 2

Effect of IGF-I and EGF on estrogen-mediated proliferation in breast cancer cells in serum-free media on collagen I. DNA content of MCF-7 (A) and T47D (B) cells after 4 d treatment with EGF (5 ng/ml) , IGF-I (25 ng/ml) and/or estrogen (10 nM) in serum-free media. Cells were plated on collagen I (Col I).). Each value represents the mean ± sem from 2 experiments. *, $P<.05$ that growth factor treated groups have a higher DNA content than control groups. **, $P<.05$ that estrogen+IGF-I+EGF group is greater than IGF-I+EGF group.

Figure 3

Effect of estrogen on proliferation of breast cancer cells on different ECM proteins in the absence of serum. DNA content of MCF-7 (A), T47D (B) and MDA MB231 (C) cells following 4 d culture in estrogen containing serum-free media was determined. Serum-free media contained 5 ng/ml EGF and 25 ng/ml IGF-I. White bars=control treatment, black bars=10 nM estrogen. Cells were plated on poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Each value represents the mean ± SEM of 3 experiments in panels A and B. Panel C is data from a representative experiment with means and error bars of replicates within experiment. *, $P<.05$ that estrogen treated groups have a higher DNA content than control groups.

Figure 4

Estrogen induced ^{3}H-thymine incorporation in breast cancer cells pretreated with the antiestrogen ICI 182,780 on different ECM proteins. MCF-7 (A), T47D (B), or MDA MB231 (D) cells were pretreated with 100 nM ICI 182,780 in serum-free media, or T47D cells in 5% charcoal stripped FBS (C), for 48 hours, followed by no treatment (Con) or estrogen (10 nM) treatment for 20 hours. Cells were plated on collagen I (Col I), laminin (LM), or fibronectin (FN). White bars=control, black bars=10 nM estrogen. Cells were subsequently labeled with ^{3}H-thymidine for 2 hours and CPM/ug DNA determined. All panels are representative experiments, with error bars of at least 3 replicates/treatment. All experiments have been repeated with similar results. *, $P<.05$ that estrogen treated groups are greater than control treated groups.

Figure 5

ER concentration of MCF-7 and T47D cells on different ECM proteins. Specific ^{3}H-estrogen binding in MCF-7 and T47D in serum-free media cultured on collagen I (Col I), laminin (LM), or fibronectin (FN) was determined. Serum-free media contained 5 ng/ml EGF and 25 ng/ml IGF-I. Each value represents the mean +/- SEM from a representative experiment with at least 3 replicates/treatment. All experiments were repeated with similar results. Estrogen receptor was determined by specific ^{3}H-estrogen binding and normalized to DNA content.

Figure 6

Estrogen regulation of progesterone receptor binding levels in MCF-7 cells on different ECM proteins in serum-free media. Specific ^{3}H-R5020 binding in MCF-7 in the absence of serum was determined. Media contained 5 ng/ml EGF and 25 ng/ml IGF-I. White bars=control treatment, Black bars=10 nM estrogen. Cells were plated on poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Each value represents the mean ± SEM of 3 separate experiments. Cells were cultured in the presence of estrogen for 3 d. Progesterone receptor (PgR) was determined by specific ^{3}H-R5020 binding and normalized to DNA content. *, $P<.05$ that estrogen treated groups are greater than control groups.

Figure 7

Estrogen stimulation of ERE-luciferase in MCF-7 cells on different ECM proteins in serum-free media. MCF-7 cells were cotransfected with ERE-luciferase (or TK-luciferase) and β-galactosidase plasmids. Serum-free media contained 5 ng/ml EGF and 25 ng/ml IGF-I. Cells were plated on collagen I (Col I), laminin (LM) or fibronectin (FN). ERE luciferase activity was measured 24 h after ICI 182,780 (100 nM), control, or estrogen (10 nM) treatment. Luciferase activity was normalized to transfection efficiency as measured by β-galactosidase expression/well and were expressed relative to the values that were obtained with the control TK-luciferase plasmid. Each value represents the means ± SEM of 3 separate experiments. *,$P<.05$ that estrogen treated groups are greater than control groups. **, $P<.05$ that laminin group treated with estrogen is greater than control treated group, but less than collagen I or fibronectin groups treated with estrogen.

Figure 8

Effect of estrogen on ^{3}H-thymidine incorporation of MCF10A TG1 and MCF10A TG3c cells on ECM proteins in 5% DCC FBS after 24 hour treatment. MCF10A TG1 (A) and TG3c (B) cells were plated in 5% DCC FBS on Poly-L-lysine (PLL), collagen I (Col I), laminin (LM) or fibronectin (FN). Control (C) or 10 nM estrogen (E) were added for 18 h. Cells were subsequently labeled with 3H-thymidine for 6 h and CPM/ug DNA determined. Each value represents the mean of at least 3 replicates.

Figure 9

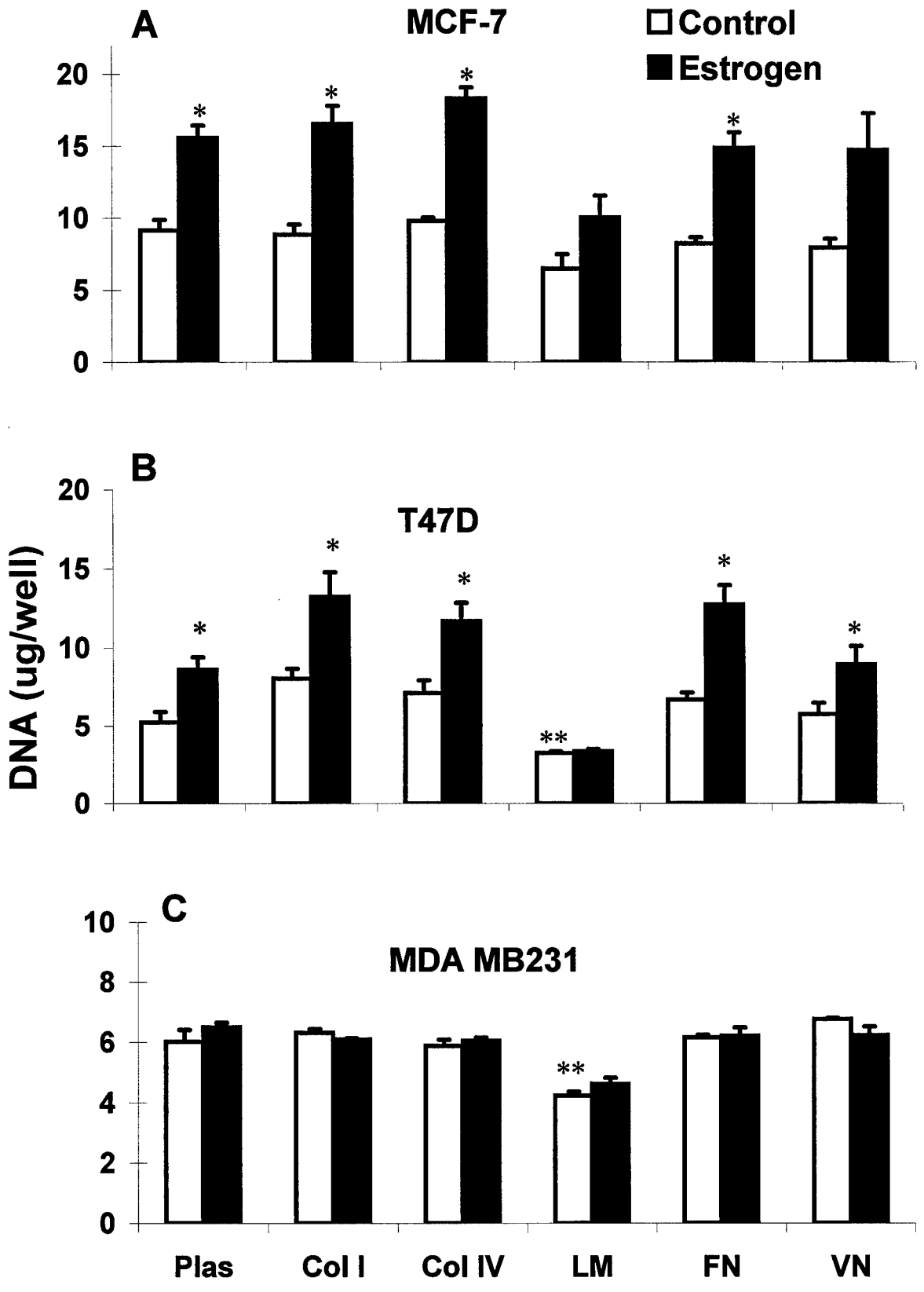
Effect of estrogen on ^{3}H-thymidine incorporation of MCF10A TG1 and MCF10A TG3c cells on ECM proteins in 5% DCC FBS after 48 hour treatment. MCF10A TG1 (A) and TG3c (B) cells were plated in 5% DCC FBS on Poly-L-lysine (PLL), collagen I (Col I), laminin (LM) or fibronectin (FN). Control (C) or 10 nM estrogen (E) were added for 42 h. Cells were subsequently labeled with 3H-thymidine for 6 h and CPM/ug DNA determined. Each value represents the mean of at least 3 replicates.

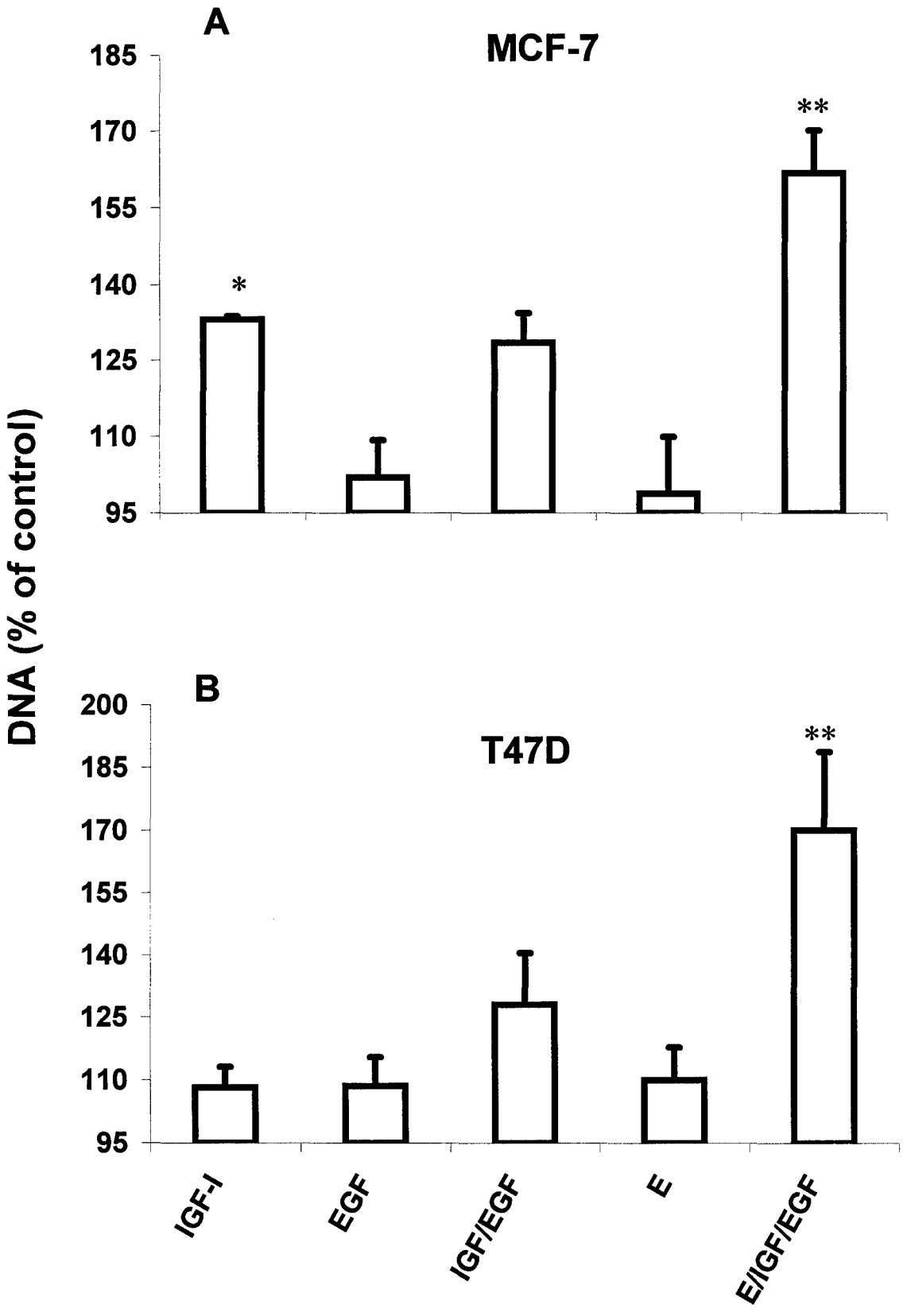
Figure 10

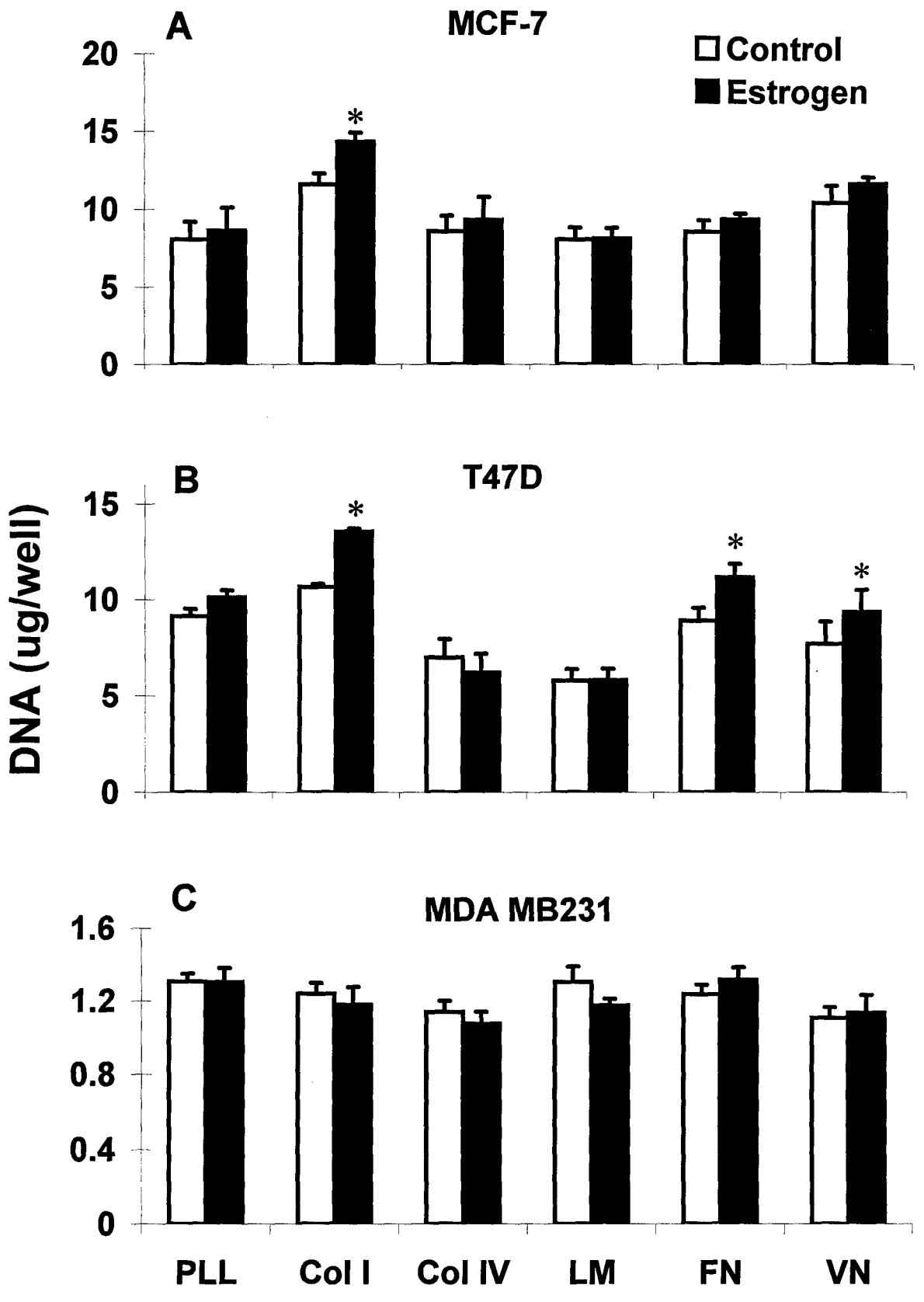
Effect of estrogen on proliferation of MCF10A TG1 and MCF10A TG3c cells on ECM proteins in 5% DCC FBS. MCF10A TG1 (A) and TG3c (B) cells were plated in 5% DCC FBS on Poly-L-lysine (PLL), collagen I (Col I), laminin (LM) or fibronectin (FN). Control (C) or 10 nM estrogen (E) were added for 4 d and DNA content determined. Each value represents the mean of at least 3 replicates.

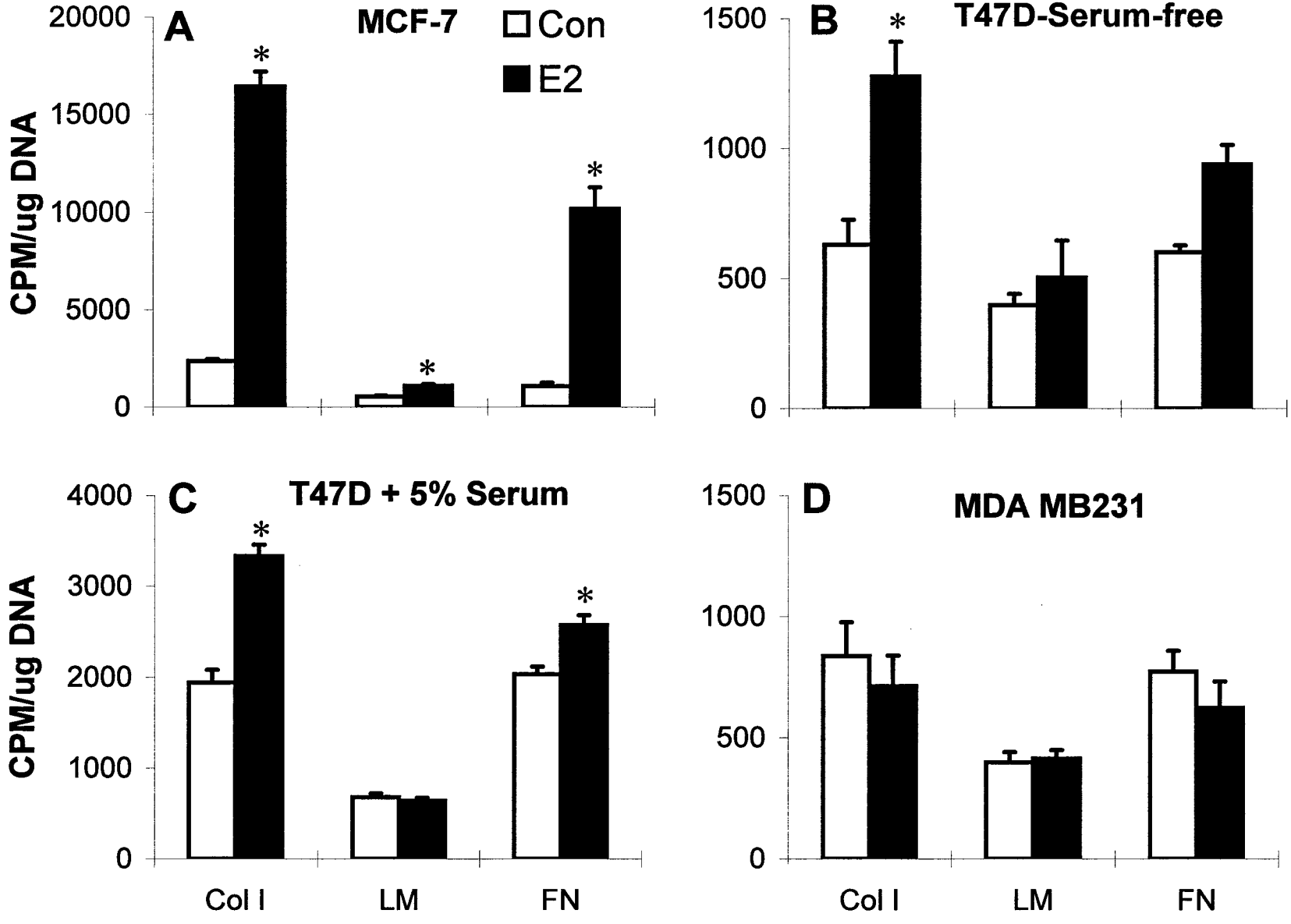
Figure 11

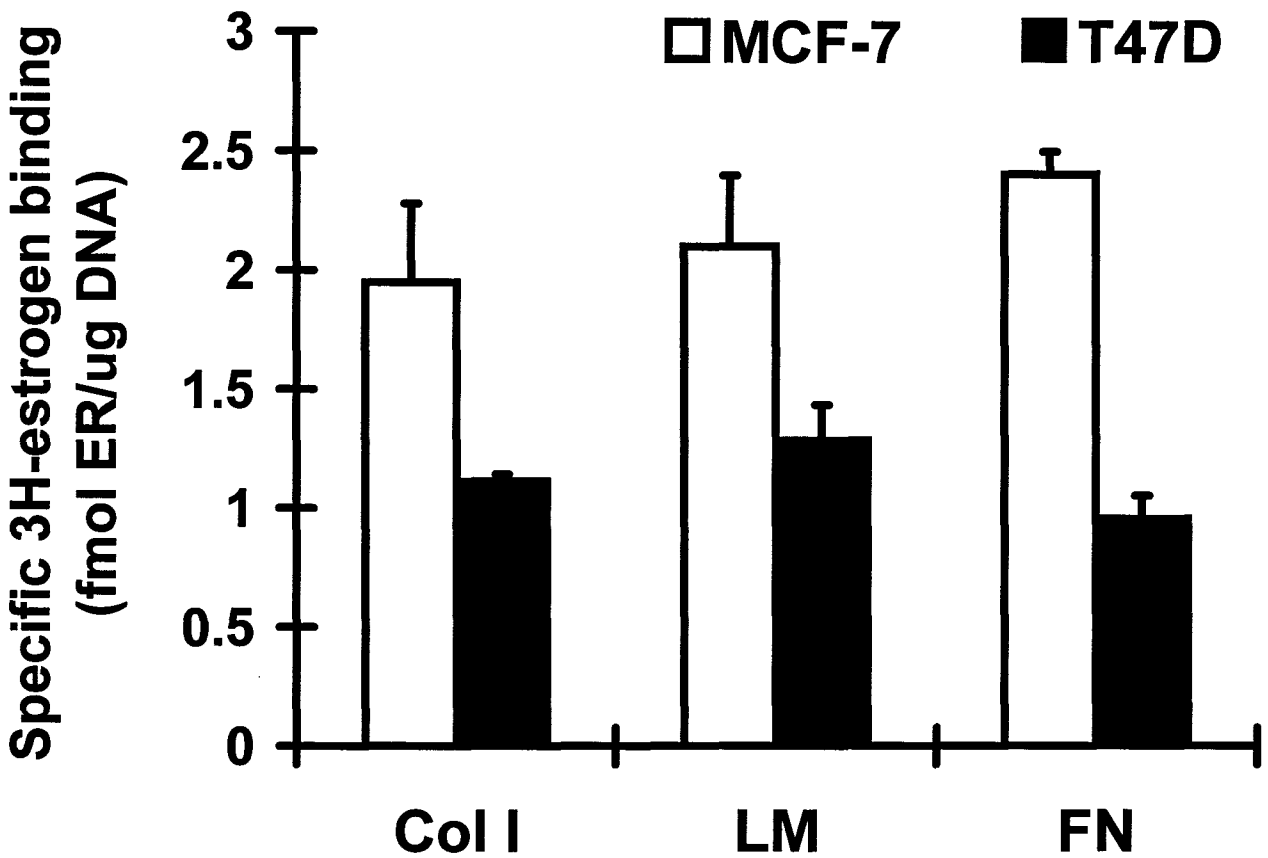
Effect of estrogen on progesterone receptor binding levels in MCF10A TG1 and MCF10A TG3c cells on ECM proteins in 5% DCC FBS. MCF10A TG1 (A) and TG3c (B) cells were plated in 5% DCC FBS on Poly-L-lysine (PLL), collagen I (Col I), laminin (LM) or fibronectin (FN). . Specific ^{3}H-R5020 binding in MCF10A TG series cells was determined. Cells were cultured in the presence of estrogen for 3 d. Progesterone receptor (PgR) was determined by specific ^{3}H-R5020 binding and normalized to DNA content.

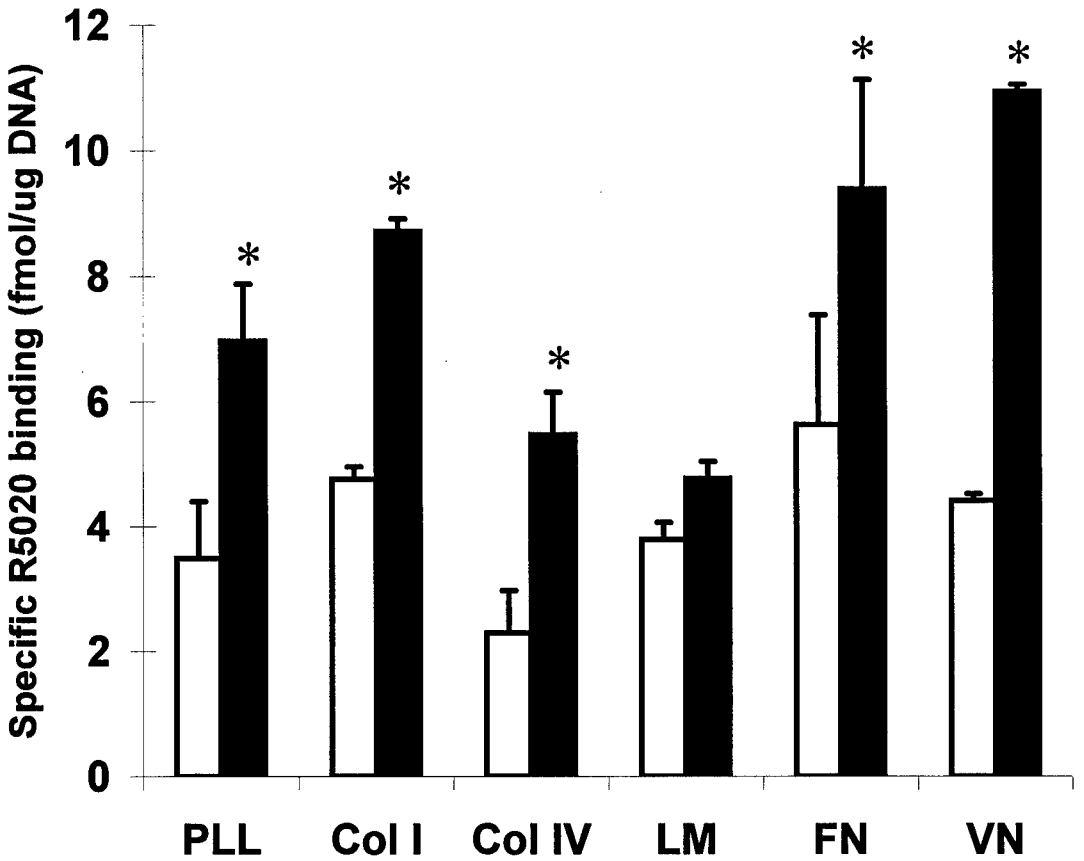

A
MCF-7
Control
Estrogen
20
15
10
5
0
B
T47D
DNA (ug/well)
C
MDA MB231
Plas
Col I
Col IV
LM
FN
VN

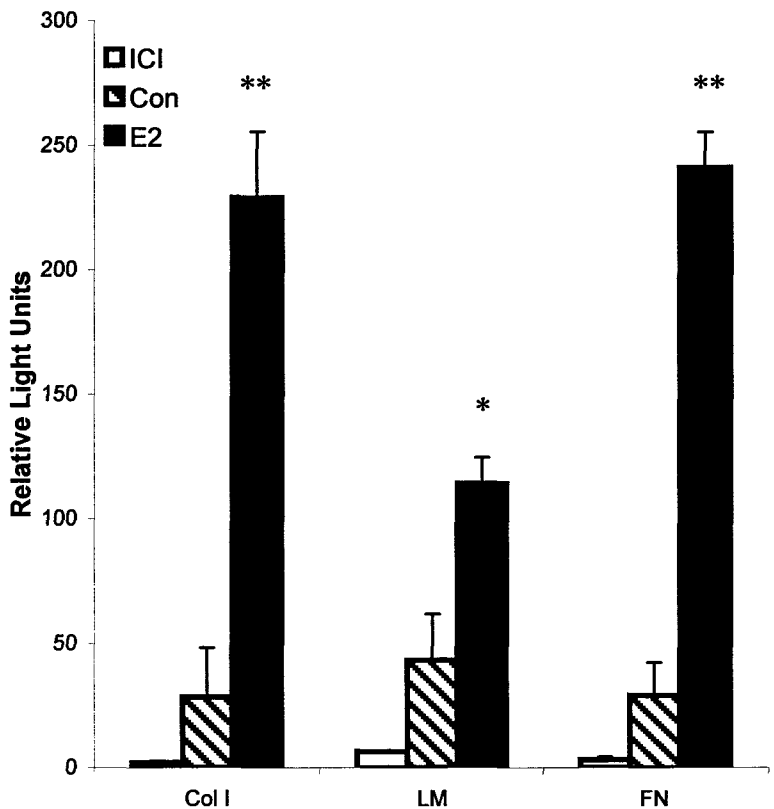

A
MCF-7
185
170
155
140
125
110
95
*
**
B
T47D
200
185
170
155
140
125
110
95
**
DNA (% of control)
IGF-I
EGF
IGF/EGF
E
E/IGF/EGF

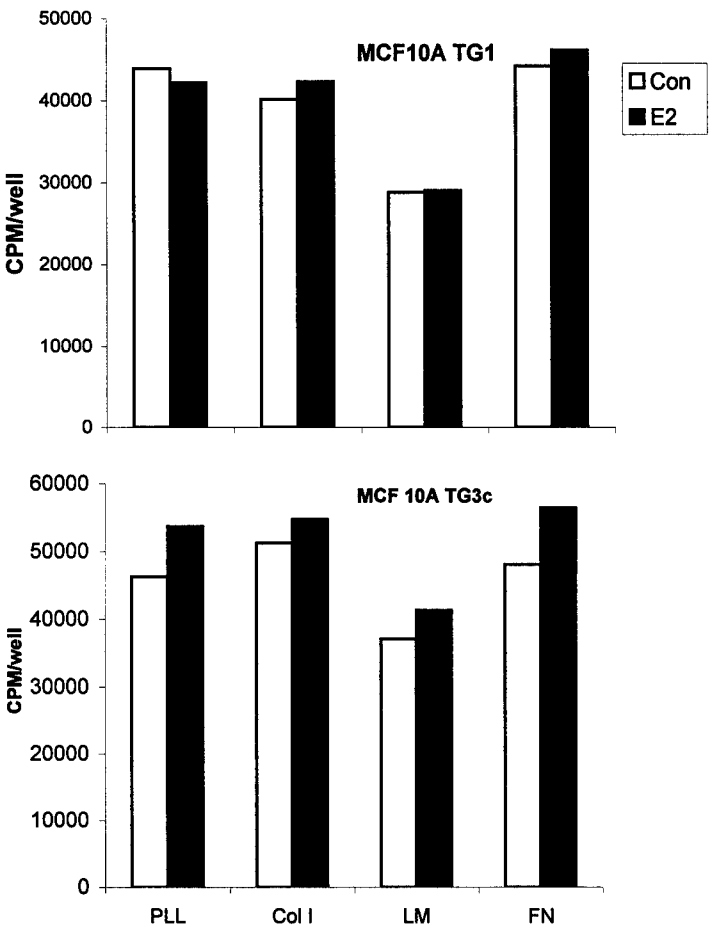

A
MCF-7
Control
Estrogen
20
15
10
5
0
*
B
T47D
DNA (ug/well)
15
10
5
0
*
*
*
C
MDA MB231
1.6
1.2
0.8
0.4
0
PLL
Col I
Col IV
LM
FN
VN

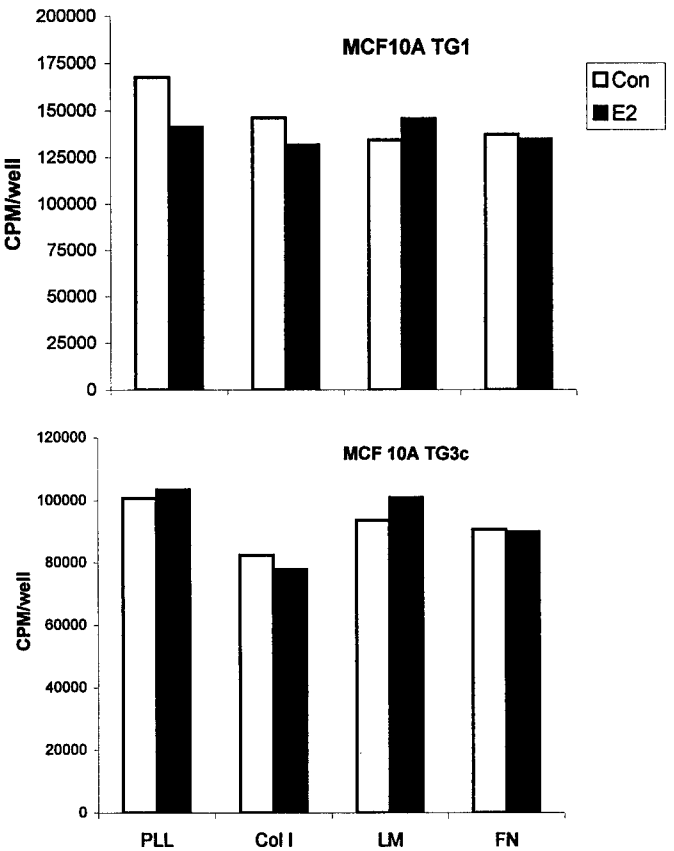

A
MCF-7
Con
E2
CPM/ug DNA
20000
15000
10000
5000
0
*
*
*
B
T47D-Serum-free
1500
1000
500
0
*
C
T47D + 5% Serum
4000
3000
2000
1000
0
*
*
Col I
LM
FN
D
MDA MB231
1500
1000
500
0
Col I
LM
FN

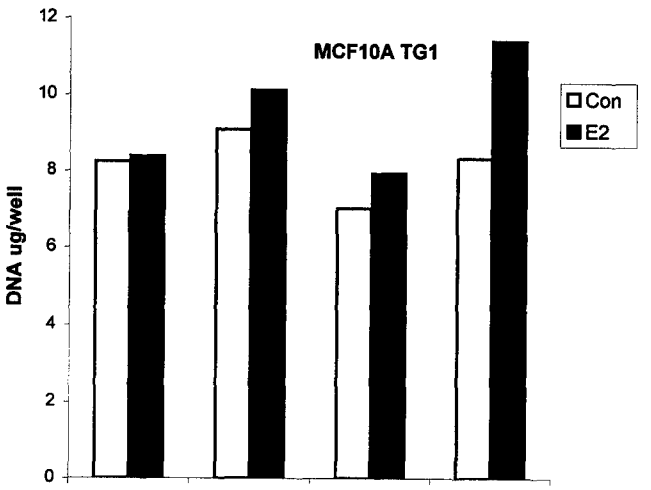

Specific 3H-estrogen binding
(fmol ER/ug DNA)
3
2.5
2
1.5
1
0.5
0
MCF-7
T47D
Col I
LM
FN

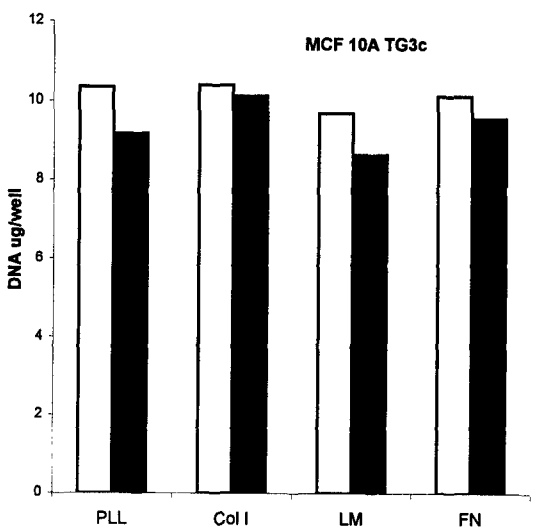

Specific R5020 binding (fmol/ug DNA)
12
10
8
6
4
2
0
*
*
*
*
*
PLL
Col I
Col IV
LM
FN
VN

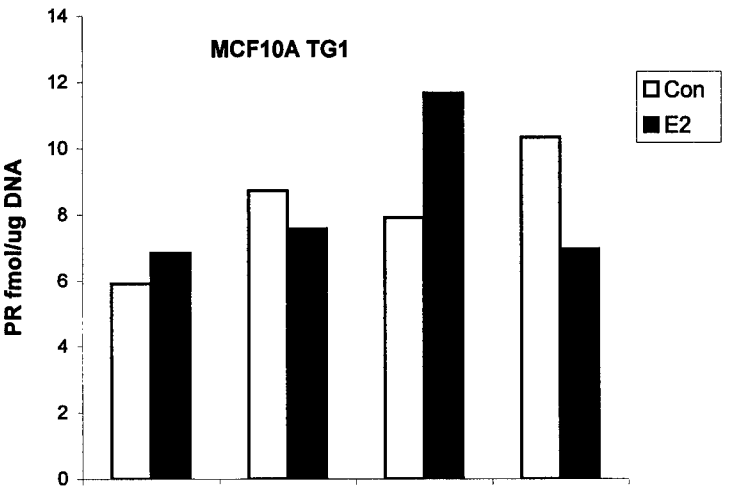

300
250
200
150
100
50
0
Relative Light Units
ICI
Con
E2
**
*
**
Col I
LM
FN

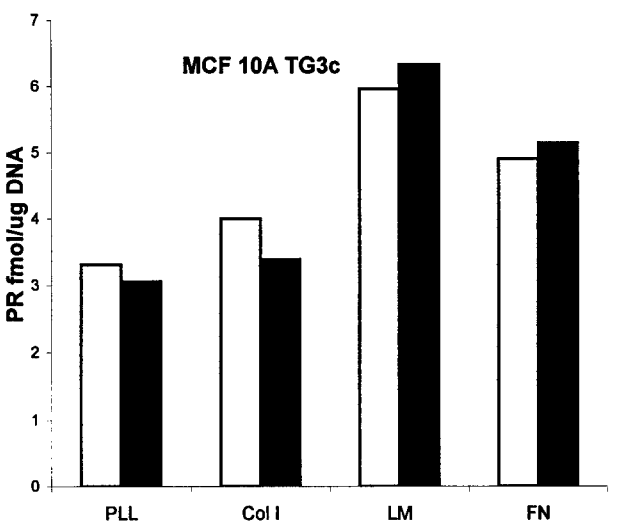

MCF10A TG1
Con
E2
CPM/well
50000
40000
30000
20000
10000
0
MCF 10A TG3c
CPM/well
60000
50000
40000
30000
20000
10000
0
PLL
Col I
LM
FN


MCF10A TG1
Con
E2
CPM/well
200000
175000
150000
125000
100000
75000
50000
25000
0
MCF 10A TG3c
CPM/well
120000
100000
80000
60000
40000
20000
0
PLL
Col I
LM
FN


MCF10A TG1
DNA ug/well
12
10
8
6
4
2
0
Con
E2


MCF 10A TG3c
DNA ug/well
12
10
8
6
4
2
0
PLL
Col I
LM
FN


MCF10A TG1
PR fmol/ug DNA
14
12
10
8
6
4
2
0
Con
E2


MCF 10A TG3c
PR fmol/ug DNA
7
6
5
4
3
2
1
0
PLL
Col I
LM
FN

Journal of Mammary Gland Biology and Neoplasia, Vol. 3, No. 2, 1998

The Role of Mammary Stroma in Modulating the Proliferative Response to Ovarian Hormones in the Normal Mammary Gland

Terry L. Woodward,[1] Jian Wei Xie,[1] and Sandra Z. Haslam[1,2]

Postnatal mammary gland development is highly dependent on the ovarian steroids, estrogen and progesterone. However, evidence from both *in vitro* and *in vivo* studies indicates that steroid-induced development occurs indirectly, requiring stromal cooperation in epithelial proliferation and morphogenesis. Stromal cells appear to influence epithelial cell behavior by secretion of growth factors and/or by altering the composition of the extracellular matrix in which epithelial cells reside. This review will discuss the requirement for stromal tissue in modulating proliferative responses to ovarian hormones during postnatal development and the potential role of the EGF, IGF, HGF and FGF[3] growth factor families. Additionally, the roles of extracellular matrix proteins, including fibronectin, collagens and laminin, will be summarized.

KEY WORDS: Epithelial-stromal interactions; mammary gland development; ductal morphogenesis; epidermal growth factor; insulin-like growth factor; hepatocyte growth factor; fibroblast growth factor; estrogen; progesterone; extracellular matrix.

INTRODUCTION

Nearly all organs and glands contain two distinct tissues: epithelium and mesenchyme (embryonic) or stroma (postnatal) (1) and their interaction is critical for the normal embryonic development of most organs. Several reviews have focused on the mechanisms by which embryonic morphogenesis of mammary gland epithelium is programmed by mammary mesenchyme, and reciprocally, how epithelium can influence the behavior of mesenchyme (1–3). However, like many reproductive tissues, the mammary gland undergoes extensive postnatal development and morphogenesis. Regulation of mammary gland development at puberty and pregnancy are highly dependent upon the ovarian steroids, estrogen and progesterone. Our laboratory and others have demonstrated, through *in vitro* and *in vivo* experiments, that epithelial-stromal interactions are critical for the initiation and maintenance of estrogen and/or progesterone responsiveness in mammary epithelial cells (4–6). The complex reciprocal interactions between epithelium and stroma that facilitate normal morphogenesis in response to steroids are likely to involve a number of different mechanisms. Here we will examine ovarian hormone-induced paracrine pathways in the mammary gland that are mediated by stroma-derived growth factors and/or extracellular matrix molecules, with a focus on studies carried out using the mouse mammary gland.

[1] Department of Physiology, 108 Giltner Hall, Michigan State University, East Lansing, Michigan 48824-1101.

[2] To whom correspondence should be addressed. e-mail: shaslam@pilot.msu.edu

[3] Abbreviations: insulin-like growth factor (IGF); hepatocyte growth factor (HGF); transforming growth factor (TGF); epidermal growth factor (EGF); fibroblast growth factor (FGF); estrogen receptor (ER); progesterone receptor (PR); estrogen response element (ERE); EGF receptor (EGFR); IGF-I receptor (IGF-1R); promegestone (R5020).

1083-3021/98/0400-0117$15.00/0

EPITHELIAL-STROMAL INTERACTIONS ARE REQUIRED FOR OVARIAN HORMONE RESPONSIVENESS IN THE MAMMARY GLAND

Ovarian Hormone Mediated Mammary Gland Development

The ontogeny, cellular distribution and regulation of estrogen receptor (ER) and progesterone receptor (PR) in relation to steroid-induced proliferation and morphogenesis in the mouse mammary gland have recently been reviewed in detail (7). Briefly, mouse mammary gland proliferation in response to exogenous estrogen is first observed in epithelial and stromal cells at three weeks of age. Ductal epithelium proliferates in response to estrogen, but not to progesterone at this time. The presence of ER is not temporally correlated with estrogen-induced cell proliferation, since both epithelial and stromal cells have immunodetectable ER by five days of age but a proliferative response is not observed until three weeks of age. Progesterone does not have a major influence on proliferation or morphological development until seven weeks of age. At this time, epithelial cells first acquire estrogen-inducible PR and progesterone-induced growth and sidebranching. The maximal synergistic response to estrogen + progesterone treatment of ovariectomized mice was observed at 10 weeks of age (8), (Fig. 1A). Stromal cells have a similar pattern of estrogen-induced proliferation, but do not exhibit a synergistic response to estrogen + progesterone (Fig. 1B).

Estrogen receptors have been detected by ligand binding and immunohistochemistry in both the epithelial and stromal compartments of the mouse mammary gland (7). Less is known about the cellular distribution of ER in other species. In the human breast, ER have not been detected in the mammary stroma by immunohistochemistry (9). However, primary cultures of human breast fibroblasts have been reported to be ER positive (10). In the case of progesterone receptors, PR have been detected in mouse mammary stroma by ligand binding studies (11). However, PR have not been consistently detected by immunohistochemical analysis [for review, see (7)]. Further studies delineating the respective roles of epithelial and stromal steroid receptors and potential differences among species are critical for determining the role of mammary stroma during mammary gland development.

It has been well documented that estrogen acts directly to stimulate mammary ductal elongation and PR expression if the pituitary is intact. In the pubertal mouse, epithelial DNA synthesis and ductal elongation were induced by locally implanted estrogen affecting only the glands that contain estrogen implants (12, 13). Additionally, it has been shown that estrogen acts locally to increase PR in the adult mammary epithelium (13). Several lines of evidence suggest that the mitogenic effect of estrogen on mammary epithelial cells is indirect. For example, in the pubertal gland, the lack of ER in the outermost layer of the end bud (cap cells), the site of proliferation leading to ductal elongation, indicates that the proliferating cells do not respond directly to estrogen (12). Additional evidence is derived from cell culture experiments that demonstrated normal mammary epithelial cells do not proliferate in response to estrogen regardless of the composition of the medium with respect to serum, hormones, growth factors or extracellular matrix proteins (4, 6, 14, 15). Studies of mammary gland development in ER knockout mice have provided additional insights into the role of mammary stroma in mediating estrogenic effects. In ER knockout mice only a rudimentary mammary gland is present at birth and no further development occurs postnatally (16). Since ER are present in both the epithelial and stromal cell compartments, it was not clear whether the lack of mammary gland development in the ER knockout ($ER^{-/-}$) mice was due to the absence of either epithelial or stromal cell ER. To address this question Cunha *et al.* (17) performed surgical recombinations of wild-type $ER^{+/+}$ epithelium and $ER^{-/-}$ stroma or $ER^{-/-}$ epithelium and wild-type $ER^{+/+}$ stroma, and transplanted these tissues under the kidney capsule of athymic nude mice. Under these conditions, $ER^{-/-}$ epithelium was able to undergo ductal morphogenesis only when grown in association with wild-type $ER^{+/+}$ stroma. Conversely, wild-type $ER^{+/+}$ epithelium did not exhibit ductal morphogenesis when combined with $ER^{-/-}$ stroma. These results provide compelling support for the hypothesis that estrogen-dependent mammary ductal proliferation is mediated through the paracrine action of estrogen-induced stroma-derived factors.

In Vivo Evidence of Stromal Influences

Direct evidence supporting the requirement for stromal tissue in the acquisition of steroid responsiveness in postnatal mammary epithelium has come

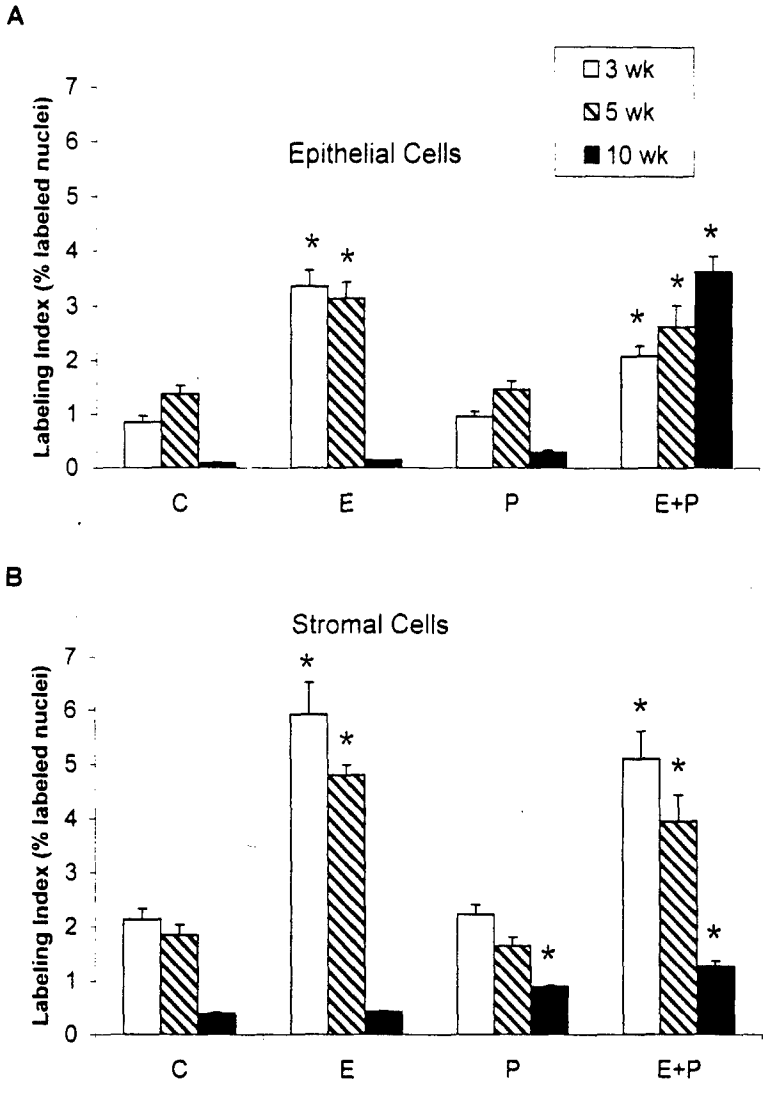


Fig. 1. Effects of ovarian hormones on DNA synthesis in mammary epithelium and stroma of mice at 3-, 5-, and 10-weeks of age. The labeling index was determined by ^{3}H-thymidine DNA histoautoradiography in ovariectomized mice 48 hours after injection of NaCl (C), 1 μg estrogen (E), 1 mg progesterone (P) or estrogen + progesterone (E+P). (A) Labeling index of ductal epithelium; (B) Labeling index of stromal cells. [Note: DNA synthesis in 5-week animals was greater at 24 hours following steroid injection than at 48 hours, so the 24 hour data is presented. *$P<0.05$, that hormone-injected groups have significantly higher labeling index than control groups.] [Adapted from Ref. 8, by permission from *Endocrinology*.]

from epithelial-stromal tissue recombination studies. Our laboratory has examined the role of mammary stroma in inducing epithelial estrogen-responsiveness (5). Adult mammary stroma (mammary fat pad) precociously induced estrogen-dependent PR in immature epithelium. That this effect was due to the adult stroma and not the adult systemic environment was shown by the observation that PR induction occurred in the adult stroma + immature epithelium tissue recombinants regardless of whether they were transplanted into immature or adult host mice (Fig. 2), (5). Mature epithelium, on the other hand, expressed significantly less estrogen-inducible PR, when recombined with immature stroma. These findings indicate that stroma not only governs the acquisition, but also the maintenance of estrogen-responsiveness in mammary epithelium.

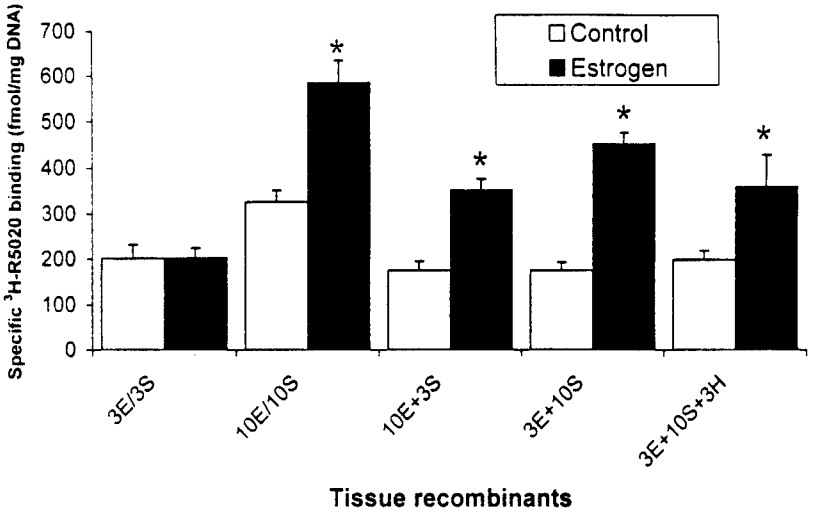


Fig. 2. Effect of mammary stroma on estrogen induction of epithelial PR, *in vivo*. Mammary epithelium from 3-week-old mice was surgically recombined with epithelium-devoid mammary stroma (cleared fat pad) of 3-week-old mice (3E/3S) or 10-week-old mice (3E + 10S), or epithelium from 10-week-old mice was recombined with stroma of 3-week-old mice (10E + 3S) or 10-week-old mice (10E/10S). To determine if systemic hormones/growth factors in host 10-week-old mice influenced the results, surgical recombinants of 10-week-old stroma and 3-week-old epithelium were transplanted onto the abdominal walls of 3-week-old host mice (3E + 10S + 3H). Two weeks after receiving transplants, the mice were ovariectomized for 1 week, injected with 1 μg estrogen or injection vehicle and 24 hours later the transplants were assayed for specific ^{3}H-R5020 binding. * $P < 0.01$, for the difference between estrogen-treated mice and control mice. [Adapted from Ref. 5, by permission from *Endocrinology*.]

Since mammary epithelial cells are ER positive for 7 weeks before they acquire estrogen-inducible PR, stroma is not facilitating this response to estrogen by inducing ER. It is more likely that stromal tissue facilitates PR induction either by releasing an inhibition of this pathway, and/or by potentiating or amplifying the pathway.

Other tissue transplantation experiments have also shown that adipose stroma is required for the normal ductal development that occurs at puberty (18). Furthermore, when mammary epithelium embedded in a collagen gel matrix is transplanted *in vivo*, spike shaped outgrowths occur, similar to epithelial growth in type I collagen gels *in vitro*. However, when the epithelial outgrowths contact adipose stroma, normal end buds form (19). Mammary gland development can also be potentiated by non-mammary adipose stroma, suggesting that adipose-specific stromal-epithelial interactions may provide an appropriate environment to facilitate estrogen-induced mammary gland development. However, if non-mammary adipose tissues contain ER, then these effects of adipose stroma may be mediated via estrogen. Elliot *et al.* (20) found that the rate of *in vivo* proliferation of a murine mammary carcinoma cell line (SP1) in response to estrogen was significantly higher when transplanted into adipose tissue than when transplanted subcutaneously. In particular, the SP1 cells maintained ER longer when transplanted into adipose tissue, suggesting that adipose tissue may influence estrogen action in the epithelial cells. Adipocyte involvement has also been implicated in estrogen-mediated proliferation of normal mouse mammary epithelium (21). In this case, estrogen-induced adipocyte proliferation was shown to precede epithelial cell proliferation in adult ovariectomized mice by 24–48 hours. These effects do not seem to be consistent across species, since others have found no proliferation of mature adipocytes either before or after estrogen treatment (22). Proximity of stromal tissue to epithelium is likely critical as several studies have implicated mammary fibroblasts adjacent to ducts as the mediators of stromal influences, since mammary fibroblasts residing close to epithelium appear to be more responsive to the proliferative effects of estrogen than nonadjacent stromal cells (22, 23).

A major problem in analyzing and interpreting the effects of mammary stroma, is the heterogeneity of cell types present in the stromal fat pad. At a mini-

mum, the mammary stroma contains mesenchymal cells, adipocytes, fibroblasts, pericytes, endothelial cells, and mast cells. Many of these cell types have been reported to have effects on the epithelium (24). However, more often than not the identification of specific cell types has relied on morphological criteria, and has not been supported by the use of cell-specific markers. Thus, in order to determine the contributions of the various stromal cells to mammary gland development, rigorous attention must be paid to the identification of specific cell types.

In Vitro Evidence of Stromal Influences

Whereas whole animal studies have demonstrated an absolute requirement for epithelial-stromal interactions in postnatal mammary gland development, tissue and cell culture models have been useful to elucidate the underlying mechanisms of cell-cell interactions and the cell types involved. When mammary glands of mature mice are cultured in whole organ culture, the original glandular architecture and epithelial and stromal tissue components are maintained. Within this system estrogen induction of PR (25) and in some instances, estrogenic stimulation of alveolar structures (26) have been demonstrated. In contrast, isolated mammary epithelial cells in culture failed to respond to estrogen (4, 6, 14, 15). However, when epithelial and stromal cells were combined in co-culture systems, estrogen-induced epithelial cell proliferation was regained (4, 6). McGrath originally reported that close contact with stromal fibroblasts was required to obtain an estrogen-induced proliferative response in epithelial cells (6). We extended that observation to show that estrogen-dependent proliferation of mammary epithelial cells required the presence of live fibroblasts, either in high numbers or in direct contact with epithelial cells (Fig. 3A), (4). Irradiated or glutaraldehye-killed fibroblasts or conditioned media from estrogen-treated fibroblasts did not mediate estrogen-induced epithelial cell proliferation (4). It is possible that conditioned media may have been ineffective because the paracrine signal was labile, inactivated by proteolysis or sequestered by binding proteins. Conversely, close contact with live fibroblasts may have been effective because: 1) the fibroblast-derived factor(s) only acts over a short distance due to low concentration or inactivation by binding proteins or proteolysis, 2) fibroblasts secrete extracellular matrix molecules that attach to the dishes immediately surrounding the live fibroblasts, but are not secreted into the media, or 3) the production of fibroblast-derived factor(s) is induced by the presence of epithelial cells. These *in vitro* findings agree with *in vivo* observations in that only stromal cells in close contact with epithelium proliferate in response to estrogen (22, 23), suggesting that the interactions between epithelial cells and fibroblasts *in vivo* are also reciprocal and only occur over relatively short distances.

Interestingly, we have found that stroma-dependent estrogen induction of cell proliferation can be dissociated from induction of PR *in vitro* (5). Estrogen-induction of PR in epithelial cells did not require the presence of live fibroblasts. In fact glutaraldehyde-killed fibroblasts, irradiated fibroblasts, conditioned media from estrogen-treated fibroblasts or culture of epithelial cells on type I collagen were all effective (Fig. 3B). These observations imply that more than one pathway or mechanism is involved in stromal regulation of estrogen action in epithelial cells. In this regard, we have proposed that stromal cells could influence mammary epithelial cells both by the production of soluble factors and/or specific extracellular matrix molecules (5, 15).

While these described cell culture studies have provided some important insights into the nature of epithelial-stromal cell interactions and ovarian hormone responsiveness, they were carried out using serum or impure supplements (serum albumin, collagen, fetuin, matrigel, pituitary extracts). Because of the numerous growth factors and other undefined potential growth promoting or growth inhibiting components present under these conditions, our understanding of the specific contribution of stromal cells and the underlying mechanisms has been limited.

MECHANISMS OF STROMAL INFLUENCES ON OVARIAN HORMONE RESPONSIVENESS

In the following sections we will review important advances in our understanding of the role of stroma-derived growth factors and ECM components as mediators/modulators of ovarian hormone responses in mammary epithelial cells. We will outline their cellular distribution and developmental regulation; regulation by and interactions with ovarian hormones; and the effects on mammary gland proliferation and morphogenesis.

A. Growth Factors

1. Epidermal Growth Factor (EGF) Family. In the pubertal gland, proliferation of the cap cell layer

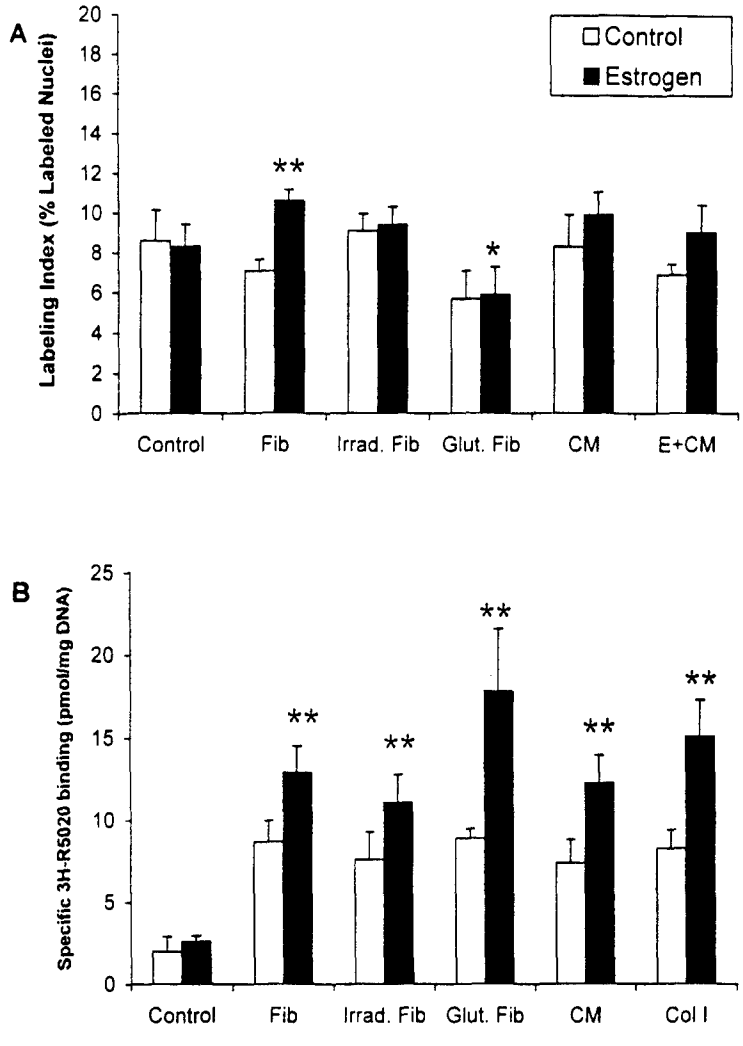


Fig. 3. Effect of fibroblast treatment on estrogen-dependent stimulation of PR or cell proliferation in epithelial cell primary culture. Epithelial cells were either cultured alone (Control), or with mammary fibroblasts that were untreated (Fib), irradiated with ^{60}Cobalt (Irrad. Fib), or killed with Glutaraldehyde (Glut. Fib), conditioned media from fibroblasts (CM), conditioned media from fibroblasts cultured in the presence of 20 nM estrogen (E + CM), or plated on collagen I (Col I). Epithelial cells or fibroblasts were plated separately in 2 cm^2 wells at a density of 2×10^5 cells/cm^2 in 5% FBS. (A) ^{3}H-thymidine incorporation (labeling index) in epithelial cells. Cells were plated with or without 20 nM 17β-estradiol, and labeling index was determined after 5 days of culture. (B) PR content in epithelial cells. At confluency the cultures were treated with 20 nM 17β-estradiol and assayed for specific ^{3}H-R5020 binding 3 days later. $*P < 0.05$, that specific R5020 binding is less than that for all other cultures. $**P < 0.05$, that labeling index on specific R5020 binding is greater in estrogen treated cells than control treated cells. [Adapted from Ref. 4, by permission from *Cancer Res.*]

of the end bud produces ductal growth and elongation. Although estrogen stimulates cap cell proliferation, ER are present in the ductal epithelium and in surrounding stromal cells, but not in the cap cells (12, 13). The cellular distribution of ER has led investigators to postulate that mitogenic effects of estrogen are indirect and may be mediated by estrogen-induced growth factors from the adjacent epithelial and/or stromal cells. Mammary derived EGF and TGF (Transforming Growth Factor)-α are believed to be potential mediators of estrogenic effects. EGF protein is localized in the luminal epithelium of the ducts, in the stromal cells adjacent to the epithelium and the interior cells of the end bud, but not in the actively proliferating cap cell layer (27, 28). TGF-α has been localized to the cap cell layer and in stromal fibroblasts around the subtending ducts (27). Because TGF-α transcripts in cap cells are very low during puberty (27) and EGF produced in the adjacent stroma or in luminal epithelial cells is present at higher levels, EGF appears to be the better candidate for the mediator of cap cell proliferation. That EGF mediates the mitogenic effect of estrogen is supported by our recent finding that the effect of estrogen on end buds was blocked by co-implantation of a neutralizing antibody specific for EGF (28). It has not been determined if adjacent epithelial cells, stromal cells or both mediate the paracrine effect of EGF on cap cell proliferation. The epithelial-stromal tissue recombination studies with ER knockout mice, in which estrogen-induced epithelial proliferation required only an ER positive mammary stroma, supports the concept that estrogen-induced paracrine effects may be mediated by stromal cells (17).

TGF-α and EGF effects on mammary development have been demonstrated by implantation of these growth factors in Elvax P40 slow release pellets (28–31). Using this technique we observed that, in addition to its stimulatory effects on epithelial cell proliferation, EGF also enhanced and/or mediated estrogen-induced PR expression in mammary epithelial cells (28). In the case of the pubertal gland, EGF combined with estrogen treatment caused precocious induction of epithelial PR. In adult mice, EGF alone was able to increase epithelial PR. The ability of estrogen to increase epithelial PR in both pubertal and adult glands could be blocked by co-implantation of a neutralizing antibody specific for EGF. Thus, EGF may mediate both estrogen-induced proliferation and epithelial PR. In the same study, we found that the endogenous EGF content of the adult gland was five-fold higher than that of the pubertal gland. Of particular interest was our finding that estrogen treatment of ovariectomized pubertal or adult mice resulted in increased EGF levels only in the stromal cells of adult mice. One interpretation of these results is that the acquisition of epithelial PR that occurs during the transition from puberty to sexual maturity is dependent upon an increased concentration of stroma-derived EGF. The increased EGF content of the adult mammary stroma may also explain why transplantation of immature epithelium to mature stroma results in the precocious acquisition of estrogen-inducible epithelial PR (5).

An analysis of EGFR distribution can provide relevant information concerning the target cells for EGF action in the mammary gland. In pubertal mice, EGFR have been localized to cap cells, myoepithelial cells and stromal cells surrounding ducts (29, 32). EGFR are also present in epithelial and stromal cells in the adult gland (32). EGFR localization, with regard to specific mammary structures, has not been analyzed in the adult gland. Since both epithelial and stromal cells possess EGFR, both cells types could be targets of EGF action. Further studies are required to elucidate the specific effects of EGF and TGF-α in epithelial and stromal cells.

2. Insulin-like Growth Factor (IGF). IGFs are produced systemically, mostly from the liver, and in target tissues (33). In the human breast, *in situ* hybridization studies have revealed that mammary gland-derived IGF-I is produced by stromal cells (34). IGF-I has also been localized to mammary stroma in the cow, where IGF-I mRNA levels were 14 to 25-fold higher in the stroma than in the epithelium (35). It remains to be determined if IGF-I is produced primarily by stromal cells in the mammary glands of other species. Preliminary results from immunohistochemical studies of IGF-I protein localization in the mouse mammary gland indicate that IGF-I is localized in stromal cells in the immature gland, but with increasing age IGF-I is also detected in the epithelium (unpublished observations, S. Z. Haslam).

Recent data from rat studies have provided some insight into estrogen/IGF-I interactions in the normal mammary gland. In the immature rat, IGF-I mRNA levels in the mammary gland were regulated by pituitary growth hormone (36). In ovariectomized, hypophysectomized rats implanted with slow release capsules containing hormones or growth factors, estrogen alone had no independent effect on terminal end bud development (36). IGF-I also had little or no inde-

pendent effect on mammary gland development. Synergistic stimulation of end bud and alveolar proliferation occurred only when IGF-I and estrogen were implanted together. The synergistic effect of estrogen was not due to stimulation of IGF-I production (36).

Most IGF-I is bound to high affinity binding proteins that modulate biological activity (33, 37). Des(1–3) IGF-I is a truncated form of IGF-I with reduced affinity for binding proteins. Implants of estrogen + des(1–3) IGF-I were four to five-fold more effective than IGF-I + estrogen in stimulating mammary gland development in the rat, suggesting that IGF binding proteins influence the synergistic actions of estrogen and IGF-I (36). Studies of cultured normal and cancerous mammary epithelial cells indicate that these cells secrete IGF binding proteins that can regulate their proliferation (38, 39). Additionally, estrogen and antiestrogens can influence the secretion of these binding proteins, thereby altering proliferation by an IGF mediated pathway. Thus, it is possible that the synergistic effect of estrogen is being mediated by regulation of binding protein production in the epithelium. However, since ER are present in both epithelial and stromal cells, further studies will be required to determine the contribution of epithelium and stroma to production of IGF-I binding proteins.

Another point of interaction between estrogen, progestins and IGF-I is the regulation of IGF-I receptors (IGF-1R). In normal human breast tissue transplanted to athymic mice, IGF-1R mRNA was increased two- to three-fold by estrogen treatment, and progesterone treatment decreased IGF-1R by approximately 50% (40). Thus, IGF-1R can be regulated by ovarian hormones and may play an important role in estrogen and IGF-I-induced morphogenesis. Clearly, further studies are needed to examine the cellular distribution and regulation by estrogen and/or progesterone of IGF-I, its receptors and binding proteins, in order to determine the role of this stroma-derived growth factor in mediating mammary gland development.

3. Hepatocyte Growth Factor (HGF). Hepatocyte growth factor (HGF)/scatter factor and its receptor c-*met* are also candidates for mediators of epithelial-stromal interactions in the mammary gland because of their respective cellular localizations, developmental regulation and possible regulation by estrogen. Recently, the temporal and spatial patterns of HGF and c-*met* expression have been analyzed during mammary gland development in the mouse and rat by several laboratories (41–44). In the rat, HGF and c-*met* mRNA levels were abundant in the nulliparous gland, decreased during pregnancy and were re-expressed during lactational involution (41). In the mouse mammary gland, Yang *et al.* (43) found a pattern of HGF expression similar to the rat. Using *in situ* hybridization, HGF transcripts were localized to a layer of stromal cells adjacent to the ductal epithelium, whereas, c-*met* transcripts were localized to basal epithelial cells in ducts. Niranjan *et al.* (42) have confirmed the localization of c-*met* transcripts to epithelial or myoepithelial cells and HGF transcripts to fibroblasts derived from mouse and human mammary glands. HGF was reportedly secreted by 3T3-L1 adipocytes, raising the possibility that this growth factor may also be produced by mammary adipocytes (45). Collectively, these data suggest that HGF is primarily expressed in stroma and c-*met* in epithelium coordinately during periods of maximal growth in the nulliparous gland and during remodeling at postlactational involution.

HGF has been shown to alter dramatically the growth and morphology of mammary epithelium in primary culture, cell lines and organ culture. It is a potent mitogen for mouse and human mammary epithelial cells, increasing proliferation and branching morphogenesis (42). The effect of HGF on branching morphogenesis of mammary epithelial cells is reviewed in detail in this issue (44).

Estrogen regulation of HGF has been reported in the mouse ovary (46) and two potential estrogen response elements (ERE) are present in the promoter of the HGF gene (47). In another study a single injection of estrogen enhanced transcription of HGF in the mouse ovary; the effect was accompanied by binding of the estrogen receptor complex to *cis*-acting ERE elements (46). At present, estrogen regulation of HGF has not been demonstrated in the mammary gland. C-*met* mRNA levels have been shown to be down-regulated by prolactin and upregulated by hydrocortisone in a murine mammary cell line, TAC-2 (41, 48). However, no effects of exogenously added estrogen or progesterone were observed (41). Since these studies were carried out in serum-containing medium, it is not possible to rule out effects due to the presence of estrogen and progesterone in the serum. Thus, further studies are needed to investigate the possible regulation of HGF and c-*met* by ovarian hormones.

4. Fibroblast Growth Factor (FGF). FGFs are one of the largest families of growth and differentiation

factors for stromal cells (49). Coleman-Krnacik and Rosen (50) examined the temporal and spatial expression of FGFs during mouse mammary gland development. FGF-1, 2, 4, and 7 transcripts were expressed during ductal development, in both pubertal and adult mice. FGF-1 (acidic or aFGF) and FGF-2 (basic or bFGF) expression decreased during pregnancy and lactation. By enzymatically separating mammary cells into epithelium enriched or fibroblast enriched fractions, they determined that stromal cells expressed FGF-2 and epithelial cells expressed FGF-1. Comparing the relative expression of FGFs in mammary fat pads devoid of epithelium with the expression in epithelial or stromal cell fractions from intact glands they concluded that FGF-2 expression in the stromal cells was induced only in the presence of epithelium (50). Thus, the expression of FGF genes appears to be regulated temporally during development by epithelial-stromal interactions.

Although FGFs have been shown to be potent mitogens for mammary epithelial cells and fibroblasts in culture (51–52), the *in vivo* actions of FGFs in the mammary gland have not been well characterized. However, nearly all FGFs have been shown to promote angiogenesis (51), which is critical for normal mammary growth/differentiation, as well as, tumor growth (53). In the uterus, estrogen was shown to increase FGF-2 levels exclusively in fibroblasts thereby stimulating capillary neovascularization (54). Progesterone may inhibit FGF-2 mRNA in fibroblasts (55). Information about ovarian hormone regulation of FGFs in the mammary gland is lacking. Since estrogen is known to promote endothelial proliferation and angiogenesis in the mammary gland (22), analysis of estrogen effects on FGF and FGF receptor gene expression could provide important insights into the role of estrogen in mammary angiogenesis.

B. Extracellular Matrix Proteins

Cell-matrix interactions are critical for regulating the phenotype of many cells. Matrix proteins produced by stromal cells, including collagen I, collagen IV, laminins, fibronectins, vitronectin and tenascin, may influence growth, morphogenesis and differentiation of epithelial cells. Epithelial cells interact with these matrix molecules using cell-surface receptors including those of the integrin family. Integrins are heterodimers composed of an alpha and a beta subunit, the combination of which determines the specificity of ligand binding [for reviews see (56, 57) this issue]. There is substantial evidence that matrix proteins and their receptors, integrins, are important for alveolar development and lactational differentiation of mammary epithelial cells (58). However, much less is known about the influence of extracellular matrix proteins on mammary cell proliferation.

Most extracellular matrix proteins in the mammary gland, including major basement membrane proteins, appear to be produced by stromal cells. It has long been known that adipocytes can produce laminin and heparan sulfate proteoglycan and that fibroblasts can produce collagen I, fibronectin and tenascin (59). Keely *et al.* (60) have presented compelling evidence, from *in situ* hybridization and immunocytochemical analyses, that mammary stromal cells are primarily (and potentially entirely) responsible for the expression of collagen I, collagen IV and laminin in the mouse mammary gland. Epithelial cells did not express any of these proteins, during ductal development, pregnancy or lactation. These findings contrast with previous reports from culture studies that epithelial cells were responsible for producing laminin and collagen IV, the most abundant proteins in the basement membrane (61–63). Keely *et al.* suggested that earlier data on basement membrane components detected in culture studies could be from contaminating stromal cells or that the biosynthetic activity of epithelial cells may be altered in culture (60). In support, Streuli and Bissell (63) have found that extracellular matrix protein expression is altered in mammary epithelial cells when they are cultured on tissue culture plastic.

Analysis of the developmental regulation of extracellular matrix proteins has revealed that defined periods of increased synthesis are correlated with peak periods of ductal proliferation in the mouse mammary gland (60). Collagen I was expressed earliest, between 2 and 5 weeks of age, and again during early pregnancy. In contrast, collagen IV was expressed between 4 and 7 weeks of age and was expressed maximally during late pregnancy. Laminin was acutely expressed at 6 to 7 weeks of age, and during late pregnancy and lactation. Interestingly, the increased expression of laminin at 6 to 7 weeks of age corresponds to the age at which ductal sidebranching and alveolar bud formation can first be induced by estrogen + progesterone treatment (8). Collagen IV was localized to the basement membrane uniformly surrounding large and small ducts and alveoli and in the adipose stroma. Collagen I was detected in the stroma and in the fibrous sheath mainly surrounding larger ducts. Laminin localization was

restricted to the basement membrane and was most concentrated around growing end buds, but was not prevalent in the adipose stroma. The authors hypothesized that collagen I may be important in duct formation, whereas laminin and collagen IV may regulate the switch to alveolus formation and lactational differentiation.

It is noteworthy that the increases in collagen I, collagen IV and laminin deposition were found in close proximity to epithelial cells in association with ducts, end buds or alveoli. In light of a report by Simon-Assmann *et al.* (64), who found that epithelial cells of the gut induced mesenchymal cells to produce laminin and collagen IV, it is quite possible that mammary epithelial cells induce mammary stromal cells to express specific ECM proteins.

Evidence that duct development in the mammary epithelium is regulated by both extracellular matrix proteins and integrin expression has been obtained from cell culture studies. Taylor-Papadimitrou and her colleagues observed that culture of normal mammary epithelial cells in collagen I gels produced balls of cells with spike-like projections (65). However, branching morphogenesis was induced in these gels by co-culture of epithelial cells and fibroblasts or fibroblast-conditioned media. Bissell and her colleagues have shown that mammary epithelial cells cultured in laminin-containing gels, also developed duct-like structures (66). They found that the $\alpha 3\beta 1$ integrin (a promiscuous receptor that binds collagens, laminins and other proteoglycans) must be downregulated in order for branching morphogenesis to take place in collagen I gels. In another study the $\alpha 3$ subunit of $\alpha 3\beta 1$ integrin, and the $\alpha 6$ subunit of the $\alpha 6\beta 1$ integrin (the classical laminin receptor) and laminin-5 were all necessary to obtain branching morphogenesis on a matrigel/laminin-5 matrix (67). These findings suggest that duct development *in vivo* likely requires complex interactions between specific matrix molecules and specific integrins. However, it should be noted that both of these studies used serum-supplemented media. Because serum contains many growth stimulating/inhibiting factors as well as extracellular matrix components it is not entirely possible to sort out the contribution of these various factors to the observed responses.

Recently, we investigated the interactions between extracellular matrix proteins, collagen I, collagen IV, laminin, fibronectin, tenascin and estrogen and progestin using mouse mammary epithelial cells in a serum-free primary culture system (15). In this system, cells were plated and cultured in the absence of serum, to ensure that cellular attachment did not occur via extracellular matrix proteins present in serum (such as fibronectin and vitronectin). We found that epithelial cells responded to ovarian steroids with proliferation only when cultured on certain matrix proteins. Epithelial cells derived from adult, nulliparous mice proliferated in response to the progestin, R5020 only on fibronectin and collagen IV (Fig. 4), (15). The responsiveness to R5020 on these matrices could not be explained either by differences in ER or PR levels or by differences in cell attachment. Epithelial cells from nulliparous mice also exhibited a proliferative response to estrogen, albeit inconsistently, on fibronectin. ER levels were not well maintained in these cultures and it is possible that estrogen-induced responses require additional signals that are provided by stromal cells *in vivo* or by serum *in vitro*. These results demonstrated that specific extracellular matrix proteins, collagen IV and fibronectin, can modulate the responsiveness of mammary epithelial cells to ovarian hormones *in vitro* and may be the mediators of stromal influences on hormone responsiveness *in vivo*. Epithelial cells from pregnant mice were not responsive to estrogen or R5020, on any matrix. Thus, responsiveness to ovarian hormones was first and foremost dependent upon the inherent state of mammary gland differentiation, but within the framework of mammary gland differentiation, matrix molecules can modulate hormonal responsiveness.

With regard to matrix/R5020 interactions, it has yet to be determined which integrin(s) and signaling pathways are involved. It is probable that more than one integrin is involved, since fibronectin and collagen IV usually bind separate integrins. Others have reported similar matrix requirements for mammary tumor proliferation; fibronectin was necessary for growth factor induced proliferation in the murine cell line, SP1 (68). In this study, platelet-derived growth factor and FGF-2 induced maximal proliferation of SP1 cells on fibronectin, whereas the growth factor response on collagen I was significantly lower.

Recently, we examined the developmental and hormonal regulation of fibronectin in mammary tissue *in vivo*. Using Western immunoblot analysis of tissue homogenates, we quantified fibronectin levels in the intact mammary gland (epithelium+stroma) and in the epithelium-devoid stroma (cleared fat pad) at 3, 5, and 10 weeks of age (Fig. 5). The greatest change in fibronectin levels occurred in the intact gland (fourfold increase) between 3 and 10 weeks of age. We also found that in adult mice, ovariectomy caused a 90% decrease of fibronectin in the intact gland and a 50%

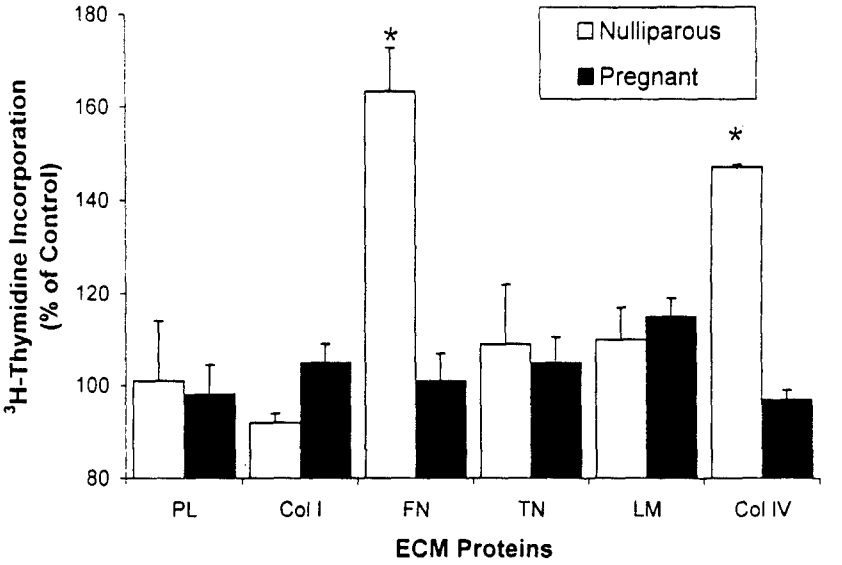


Fig. 4. Effect of R5020 and extracellular matrix (ECM) components on mammary epithelial DNA synthesis in primary serum-free cultures. Epithelial cells derived from nulliparous or pregnant mice were plated at 1.4×10^5 cells/well in 96-well plates treated with poly-L-lysine (PL), collagen I (Col I), fibronectin (FN), tenascin (TN), laminin (LM), or collagen IV (Col IV). Plating and growth media were all serum-free, and did not contain growth factors. Medium was changed at 24 hours to control or R5020 (20 nM) and ^{3}H-thymidine incorporation into DNA was determined 48 hours later. The percent increase was obtained by dividing the CPM/mg DNA of cells cultured in the presence of R5020 by the CPM/mg DNA of cells cultured in the absence of R5020. $^*P < 0.05$, that R5020 stimulated proliferation of nulliparous-derived cells on FN and on Col IV. [Adapted from Ref. 15, by permission from *Endocrinology*.]

decrease in the cleared fat pad. Treatment with exogenous estrogen increased fibronectin two-fold in the intact gland, but not in the cleared fat pad. These results demonstrated that the temporal pattern of fibronectin protein expression paralleled the temporal pattern of mammary gland maturation and the acquisition of progesterone responsiveness. Furthermore, fibronectin expression appeared to be regulated by estrogen. Thus, it is possible to speculate that fibronectin plays an important role in hormone-dependent mammary gland development *in vivo*. Since it has previously been shown that fibronectin expression occurs only in stromal cells in the mouse mammary gland (60), our results further suggest that fibronectin levels in the intact gland are subject to an inductive influence of the epithelium, as has been demonstrated in the gut (64).

Fibronectin is a major stroma-derived component of the extracellular matrix and appears to be required for normal development (69). In a number of experimental systems fibronectin has been shown to promote proliferation (70). The novel findings that fibronectin is important for estrogen and progestin-induced epithelial proliferation *in vitro* and is developmentally and hormonally regulated *in vivo* indicate that fibronectin may also be involved in mediating ovarian hormone effects *in vivo*.

In conclusion, evidence derived from extracellular matrix/integrin studies on mammary gland lactational function and our own studies on extracellular matrix /ovarian hormone effects on mammary cell proliferation, support the hypothesis that developmentally regulated alterations in matrix composition may potentiate and sustain the effects of steroids. Future studies of the developmental and hormonal regulation of integrin expression should provide important information about integrin/matrix interactions and identification of distinct signaling cascades that mediate cellular proliferation, differentiation and apoptosis in the mammary epithelium.

CONCLUSIONS

We have reviewed the modulation of ovarian hormone-dependent proliferation by mammary stroma. Two major mechanisms by which stromal cells can

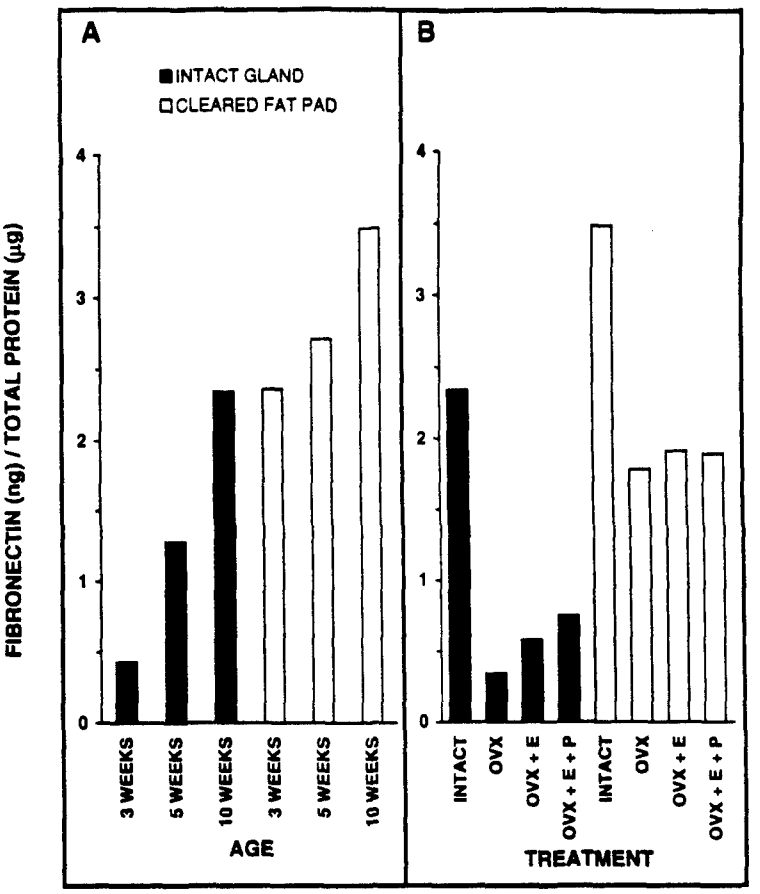


Fig. 5. Developmental and hormone-induced changes in fibronectin levels in mammary tissues of 3-, 5-, and 10-week old mice. Mammary tissues from intact mammary glands (epithelium + stroma) or epithelium-devoid cleared fat pads (mammary stroma only) were analyzed in (A) ovary intact mice at 3-, 5-, and 10-weeks of age or in (B) 10-week-old mice that were intact (Intact), or ovariectomized and treated with estradiol (E, 1 µg/mouse) or estradiol + progesterone (E + P, 1 µg and 1 mg/mouse, respectively) or with vehicle control (OVX) for 14 days prior to assay. The extracellular matrix proteins in mammary tissue homogenates were separated electrophorectically (PAGE), and quantified by PhopholImager analysis of Western immunoblots. Samples were adjusted to equal protein concentrations before loading gels. Five to six concentrations of purified fibronectin were ran on each gel to use as a standard curve for PhosphorImager quantification and to confirm the correct molecular weight of samples.

influence epithelial cell behavior have been examined: 1) the production of soluble growth factors and 2) the modification of the composition of the extracellular matrix. The interactions are bi-directional such that epithelial cells are also capable of influencing stromal cell behavior. Ovarian hormones and growth factors interact and modulate both growth factor expression and the expression of their respective receptors. These interactions appear to be important for the coordinated regulation of growth and differentiation of the normal mammary gland.

Deciphering the complex interactions involved in stromal mediation of ovarian hormone-induced proliferation in the normal mammary gland has specific relevance to understanding alterations in hormone responsiveness in breast cancer. This is especially true since a dramatic alteration in the morphological and secretory phenotype of stromal cells surrounding the tumor frequently occurs during breast carcinogenesis. Determining the mechanisms underlying epithelial-stromal cell interactions in normal development and the changes that occur during mammary carcinogene-

sis may provide a conceptual basis for novel approaches to the prevention and treatment of breast cancer.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Ahmed M. Raafat, James L. Fendrick, and Lorne J. Hofseth for manuscript review. Our appreciation is also extended to Andy S. Mienaltowski and Dr. James L. Fendrick for figure preparation. Supported by NIH grant 5R01CA40104 to Dr. S. Z. Haslam and DOD grant DAMD 17-96-1-6026 to Dr. T. L.Woodward.

REFERENCES

1. C. Birchmeier and W. Birchmeier (1993). Molecular aspects of mesenchymal-epithelial interactions. *Ann. Rev. Cell Biol.* **9**:511–540.
2. K. Kratochwil (1969). Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. *Devel. Biol.* **20**:46–71.
3. G. R. Cunha and Y. K. Hom (1996). Role of mesenchymal-epithelial interactions in mammary gland development. *J. Mam. Gland Biol. Neoplasia* **1**:21–35.
4. S. Z. Haslam (1986). Mammary fibroblast influence on normal mouse mammary epithelial cell responses to estrogen *in vitro*. *Cancer Res.* **46**:310–316.
5. S. Z. Haslam and L. J. Counterman (1991). Mammary stroma modulates hormonal responsiveness of mammary epithelium *in vivo* in the mouse. *Endocrinology* **129**:2017–2023.
6. C. M. McGrath (1983). Augmentation of the response of normal mammary epithelial cells to estradiol by mammary stroma. *Cancer Res.* **43**:1355–1360.
7. J. L. Fendrick, A. M. Raafat, and S. Z. Haslam (1998). Mammary gland growth and development from the postnatal period to postmenopause: ovarian steroid receptor ontogeny and regulation in the mouse. *J. Mam. Gland Biol. Neoplasia* **4** (in press).
8. S. Z. Haslam (1989). The ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones. *Endocrinology* **125**:2766–2772.
9. K. S. McCarty, Jr., E. Szabo, J. L. Flowers, E. B. Cox, G. S. Leight, L. Miller, J. Konrath, J. T. Soper, D. A. Budwit, W. T. Creasman, H. F. Seigler, and K. S. McCarty, Sr. (1986). Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. *Cancer Res.* (Suppl.) **46**:4244s–4248s.
10. C. Malet, A. Gompel, H. Yaneva, H. Cren, N. Fidji, I. Mowszowicz, F. Kuttenn, and P. Mauvais-Jarvis (1991). Estradiol and progesterone receptors in cultured normal human breast epithelial cells and fibroblasts: Immunocytochemical studies. *J. Clin. Endocrinol. Metabol.* **73**:8–17.
11. S. Z. Haslam and G. Shyamala (1981). Relative distribution of estrogen and progesterone receptors among the epithelial, adipose, and connective tissue components of the normal mammary gland. *Endocrinology* **108**:825–830.
12. C. W. Daniel, G. B. Silberstein, and P. Strickland (1987). Direct action of 17 beta-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. *Cancer Res.* **47**:6052–6057.
13. S. Z. Haslam (1988). Local versus systemically mediated effects of estrogen on normal mammary epithelial cell deoxyribonucleic acid synthesis. *Endocrinology* **122**:860–867.
14. W. Imagawa, J. Yang, R. Guzman, and S. Nandi (1994). Control of mammary gland development. In E. Knobil and J. D. Neill (eds.), *The Physiology of Reproduction*, Second Edition, Raven Press, Ltd., New York, pp. 1033–1063.
15. J. Xie and S. Z. Haslam (1997). Extracellular matrix regulates ovarian hormone-dependent proliferation of mouse mammary epithelial cells. *Endocrinology* **138**:2466–2473.
16. W. P. Bocchinfuso and K. S. Korach (1997). Mammary gland development and tumorigenesis in estrogen receptor knockout mice. *J. Mam. Gland Biol. Neoplasia* **2**:323–334.
17. G. R. Cunha, P. Young, Y. K. Hom, P. S. Cooke, J. A. Taylor, and D. B. Lubahn (1997). Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombination experiments. *J. Mam. Gland Biol. Neoplasia* **2**:393–402.
18. K. Hoshino. (1978). Mammary transplantation and its histogenesis in mice. In A. Yokoyama, M. Mizuno and H. Nagasawa (eds.), *Physiology of Mammary Glands*, University Park Press, University Park, Maryland, pp. 163–228.
19. C. W. Daniel, J. J. Berger, P. Strickland, and R. Garcia. (1984). Similar growth pattern of mouse mammary cells cultivated in collagen matrix *in vivo* and *in vitro*. *Devel. Biol.* **104**:57–64.
20. B. E. Elliot, S. P. Tam, D. Dexter, and Z. Q. Chen (1992). Capacity of adipose tissue to promote growth and metastasis of a murine mammary carcinoma: effect of estrogen and progesterone. *Int. J. Cancer* **51**:416–424.
21. G. Shyamala and A. Ferenczy (1984). Mammary fat pad may be a potential site for initiation of estrogen action in normal mouse mammary glands. *Endocrinology* **115**:1078–1081.
22. T. L. Woodward, W. E. Beale, and R. M. Akers (1993). Cell interactions in initiation of mammary epithelial proliferation by oestradiol and progesterone in prepubertal heifers. *J. Endocrinol.* **136**:149–157.
23. J. J. Berger and C. W. Daniel (1983). Stromal DNA synthesis is stimulated in young, but not serially aged, mouse mammary epithelium. *Mech. Aging Devel.* **2**:259–264.
24. S. Z. Haslam (1988). Cell to cell interactions and normal mammary gland function. *J. Dairy Sci.* **71**:2843–2854.
25. S. Z. Haslam, L. J. Counterman, and A. R. St. John (1993). Hormonal basis for acquisition of estrogen-dependent progesterone receptors in the normal mouse mammary gland. *Steroid Biochem.* **12**:27–34.
26. J. Barlow, T. Casey, J - F Chiu, and K. Plaut (1997). Estrogen affects development of alveolar structures in whole-organ culture of mouse mammary glands. *Biochem. Biophys. Res. Commun.* **232**:340–344.
27. S. M. Snedeker, C. F. Brown, and R. P. DiAugustine (1991). Expression and functional properties of transforming growth factor-α and epidermal growth factor during mouse mammary gland ductal morphogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **88**:276–280.
28. D. P. Ankrapp, J. M. Bennett, and S. Z. Haslam (1998). The role of epidermal growth factor in the acquisition of ovarian steroid hormone responsiveness in the normal mouse mammary gland. *J. Cell Physiol.* **174**:251–260.
29. S. Coleman, G. B. Silberstein, and C. W. Daniel (1988). Ductal morphogenesis in the mouse mammary gland: evidence supporting a role for epidermal growth factor. *Devel. Biol.* **127**:304–315.
30. B. K. Vonderhaar (1987). Local effects of EGF, α-TGF, and EGF-like growth factors on lobuloalveolar development of the mouse mammary gland *in vivo*. *J. Cell. Physiol.* **132**:581–584.

31. S. Z. Haslam, L. J. Counterman, and K. A. Nummy (1993). Effects of epidermal growth factor, estrogen and progestin on DNA synthesis in mammary cells *in vivo* are determined by the developmental state of the gland. *J. Cell. Physiol.* **155:**72–78.
32. S. Z. Haslam, L. J. Counterman, and K. A. Nummy (1992). EGF receptor regulation in normal mouse mammary gland. *J. Cell. Physiol.* **152:**553–557.
33. J. I. Jones and D. R. Clemmons (1995). Insulin-like growth factors and their binding proteins: Biological actions. *Endocrine Rev.* **16:**3–34.
34. D. Yee, S. Paik, G. S. Lebovic, R. R. Marcus, R. E. Favoni, K. J. Cullen, M. E. Lippman, and N. Rosen (1989). Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. *Mol. Endocrinol.* **3:**509–517.
35. S. D. Hauser, M. F. McGrath, R. J. Collier, and G. G. Krivi (1990). Cloning and *in vivo* expression of bovine growth hormone receptor mRNA. *Mol. Cell Endocrinol.* **72:**187–200.
36. W. Ruan, V. Catanese, R. Wieczorek, M. Feldman, and D. L. Kleinberg (1995). Estradiol enhances the stimulatory effect of insulin-like growth factor-I (IGF-I) on mammary development and growth hormone-induced IGF-I messenger ribonucleic acid. *Endocrinology* **136:**1296–1302.
37. K. Swisshelm, K. Ryan, K. Tsuchiya, and R. Sager (1995). Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. *Proc. Natl. Acad. Sci. U. S. A.* **92:**4472–4476.
38. H. Huynh, X. F. Yang, and M. Pollak (1996). A role for insulin-like growth factor binding protein 5 in the antiproliferative action of the antiestrogen ICI 182780. *Cell Growth Differ.* **7:**1501–1506.
39. H. Huynh, X. Yang, and M. Pollak (1996). Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. *J. Biol. Chem.* **271:**1016–1021.
40. R. B. Clarke, A. Howell, and E. Anderson (1997). Type I insulin-like growth factor receptor gene expression in normal human breast tissue treated with oestrogen or progesterone. *Brit. J. Cancer* **75:**251–257.
41. M. S. Pepper, J. V. Soriano, P. A. Menoud, A. P. Sappino, L. Orci, and R. Montesano (1995). Modulation of hepatocyte growth factor and c-Met in the rat mammary gland during pregnancy lactation and involution. *Exp. Cell Res.* **219:**204–210.
42. B. Niranjan, L. Buluwela, J. Yant, N. Perusinghe, A. Atherton, D. Phippard, T. Dale, B. Gusterson, and T. Kamalati (1995). HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. *Development* **121:**2897–2908.
43. Y. Yang, E. Spitzer, D. Meyer, M. Sachs, C. Neimann, G. Hartmann, K. M. Weidner, C. Birchmeier, and W. Birchmeier (1995). Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. Cell Biol.* **131:**215–226.
44. J. V. Soriano, M. S. Pepper, L. Orci, and R. Montessano (1998). Roles of hepatocyte growth factor/scatter factor and transforming growth factor-β_1 in mammary ductal morphogenesis. *J. Mam. Gland Biol. Neoplasia* **3:**133–150.
45. N. Rahimi, R. Saulnier, T. Nakamura, M. Park, and B. Elliott (1994). Role of hepatocyte growth factor in breast cancer: a novel mitogenic factor secreted by adipocytes. *DNA Cell Biol.* **13:**1189–1197.
46. Y. Liu, L. Lin, and R. Zarnegar (1994). Modulation of hepatocyte growth factor gene expression by estrogen in the mouse ovary. *Mol. Cell. Endocrinol.* **104:**173–181.
47. Y. Liu, G. K. Michalopoulos, and R. Zarnegar (1994). Structural and functional characterization of the mouse hepatocyte growth factor gene promoter. *J. Biol. Chem.* **269:**4152–4160.
48. J. V. Soriano, M. S. Pepper, T. Nakamura, L. Orci, and R. Montesano (1995). Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. *J. Cell Sci.* **108:**413–430.
49. D. Givol and A. Yayon (1992). Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J.* **6:**3362–3369.
50. S. Coleman-Krnacik and J. M. Rosen (1994). Differential temporal and spatial gene expression of fibroblast growth factor family members during mouse mammary gland development. *Mol. Endocrinol.* **8:**218–229.
51. G. D. Shipley, W. W. Keeble, J. E. Hendrickson, R. J. Coffey, Jr., and M. R. Pittelkow (1989). Growth of normal human keratinocytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. *J. Cell Physiol.* **138:**511–518.
52. L. Ronnov-Jessen and O. W. Petersen (1993). Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Lab. Invest.* **68:**696–707.
53. A. Johns, A. D. Freay, W. Fraser, K. S. Korach, and G. M. Rubanyi (1996). Disruption of estrogen receptor gene prevents 17 beta estradiol-induced angiogenesis in transgenic mice. *Endocrinology* **137:**4511–4513.
54. J. Fujimoto, M. Hori, S. Ichigo, and T. Tamaya (1996). Expression of basic fibroblast growth factor and its mRNA in uterine endometrium during the menstrual cycle. *Gynecol. Endocrinol.* **10:**193–197.
55. J. Fujimoto, M. Hori, S. Ichigo, and T. Tamaya (1997). Ovarian steroids regulate the expression of basic fibroblast growth factor and its mRNA in fibroblasts derived from uterine endometrium. *Ann. Clin. Biochem.* **34:**91–96.
56. M. M. Zutter, H. Sun, and S. A. Santoro (1998). Altered integrin expression and the malignant phenotype: the contribution of multiple integrated integrin-receptors. *J. Mam. Gland Biol. Neoplasia* **3:**191–200.
57. C. H. Streuli and G. Edwards (1998). Control of normal mammary epithelial phenotype by integrins. *J. Mam. Gland Biol. Neoplasia* **3:**151–164.
58. C. H. Streuli, C. Schmidhauser, N. Bailey, P. Yurchenco, A. P. N. Skubitz, C. Roskelley, and M. J. Bissell (1995). Laminin mediates tissue-specific gene expression in mammary epithelia. *J. Cell Biol.* **129:**591–603.
59. A. R. Howlett and M. J. Bissell (1993). The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium. *Epith. Cell Biol.* **2:**79–89.
60. P. J. Keely, J. E. Wu, and S. A. Santoro (1995). The spatial and temporal expression of the α_2 β_1 integrin and its ligands, collagen I, collagen IV, and laminin, suggests important roles in mouse mammary morphogenesis. *Differentiation* **59:**1–13.
61. M. S. Wicha (1984). Interaction of rat mammary epithelium with extracellular matrix components. *Prog. Clin. Biol. Res.* **145:**129–142.
62. G. Parry, B. Cullen, C. S. Kaetzel, R. Kramer, and L. Moss (1987). Regulation of differentiation and polarized secretion in mammary epithelial cells maintained in culture: extracellular matrix and membrane polarity influences. *J. Cell Biol.* **105:**2043–2051.
63. C. H. Streuli and M. J. Bissell (1990). Expression of extracellular matrix components is regulated by substratum. *J. Cell Biol.* **110:**1405–1415.
64. P. Simon-Assmann, F. Bouziges, C. Arnold, K. Haffen, and M. Kedinger (1988). Epithelial-mesenchymal interactions in the production of basement membrane components in the gut. *Development* **102:**339–347.
65. F. Berdichevsky, D. Alford, B. D'Souza, and J. Taylor-Papadimitriou (1994). Branching morphogenesis of human

mammary epithelial cells in collagen gels. *J. Cell Sci.* **107**:3557–3568.
66. M. L. Li, J. Aggeler, D. A. Farson, C. Hatier, J. Hassell, and M. J. Bissell (1987). Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **84**:136–140.
67. S. Stahl, S. Weitzman, and J. C. R. Jones (1997). The role of laminin-5 and its receptor in mammary epithelial cell branching morphogenesis. *J. Cell. Sci.* **110**:55–63.
68. B. Elliot, A. Ostman, B. Westermark, and K. Rubin (1992). Modulation of growth factor responsiveness of murine mammary carcinoma cells by cell matrix interactions: correlation of cell proliferation and spreading. *J. Cell. Physiol.* **152**:292–301.
69. R. O. Hynes (1994). Genetic analyses of cell-matrix interactions in development. *Curr. Opin. Genet. Devel.* **4**:569–574.
70. K. K. Wary, F. Mainiero, S. J. Isakoff, E. E. Marcantonio, and F. G. Giancotti (1996). The adapter protein Shc couples a class of integrins to the control of cell cycle progression. *Cell* **87**:733–743.